The final size of plant organs is controlled by a complex regulatory network of molecular players governed by different plant hormones. By analyzing Arabidopsis plants showing enhanced growth due to overexpression of distinct genes at the morphological and molecular level, we aimed to uncover new genes and new interactions between known genes thereby extending the regulatory network that controls plant growth.

Thesis submitted as partial fulfillment
of the requirements for the degree of
Doctor (Ph.D.) in Sciences: Biotechnology
Academic year 2012-2013

VIB

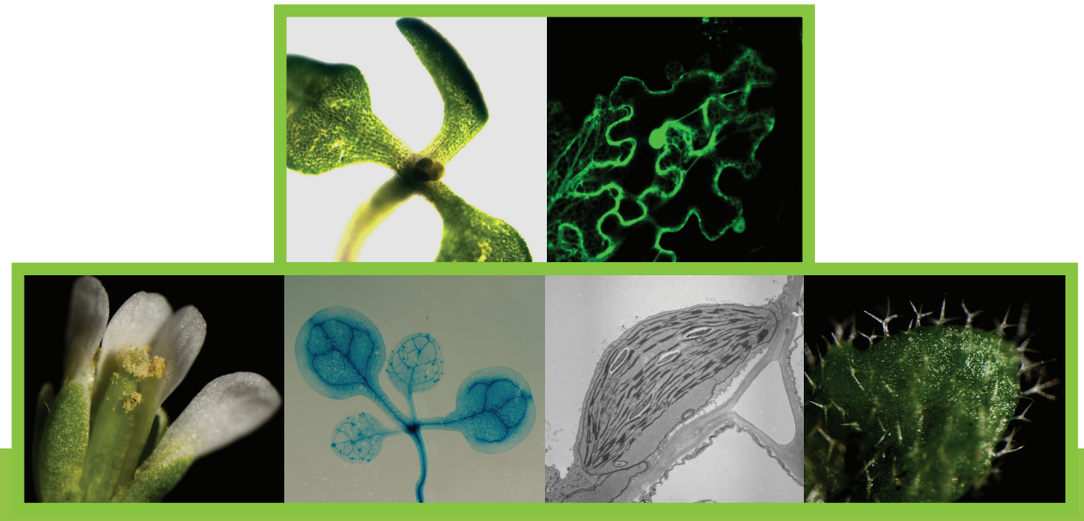
Plant Systems Biology

UNIVERSITEIT GENT

Integration of cytokinin responses and chromatin remodeling to regulate transcription during Arabidopsis shoot development

Liesbeth Vercruyssen

Integration of cytokinin responses and chromatin remodeling to regulate transcription during Arabidopsis shoot development



Liesbeth Vercruyssen

Ghent University
Faculty of Sciences
Department of Plant Biotechnology and Bioinformatics

Integration of cytokinin responses and chromatin remodeling to regulate transcription during Arabidopsis shoot development

Liesbeth Vercruyssen

Thesis submitted as partial fulfillment of the requirements for
the degree of Doctor (Ph.D.) in Sciences: Biotechnology
Academic year 2012-2013

Promoter:
Prof. Dr. Dirk Inzé

VIB – Plant Systems Biology
Systems Biology of Yield Group
Technologiepark 927, 9052 Ghent, Belgium

Cover

Top left, eight-day-old Arabidopsis Col-0 seedling containing three cotyledons and three first leaves. Top right, *35S:AN3-EGFP* localization in Tobacco epidermal cells after *Agrobacterium*-mediated leaf infiltration. Bottom left, Arabidopsis Col-0 flower. Bottom middle left, ten-day-old Arabidopsis Col-0 seedling expressing *ARR4:GUS* after overnight staining. Bottom middle right, electron micrograph of a chloroplast from *35S:GRF5* Arabidopsis Col-0 leaves at 21 days after stratification. Bottom right, close-up view of an Arabidopsis Col-0 rosette leaf.

Prof. Dr. Dirk Inzé (Promoter)
Faculty of Sciences, Department of Plant Biotechnology and Bioinformatics
Ghent University

Prof. Dr. Geert De Jaeger (Chair)
Faculty of Sciences, Department of Plant Biotechnology and Bioinformatics
Ghent University

Prof. Dr. Lieven De Veylder
Faculty of Sciences, Department of Plant Biotechnology and Bioinformatics
Ghent University

Dr. Eva Benkova
Faculty of Sciences, Department of Plant Biotechnology and Bioinformatics
Ghent University

Prof. Dr. Godelieve Gheysen
Faculty of Bioscience Engineering, Department of Molecular Biotechnology
Ghent University

Dr. Rafal Archacki
Laboratory of Plant Molecular Biology
University of Warsaw

Prof. Dr. Claire Périlleux
Laboratory of Plant Physiology
University of Liège

Prof. Dr Tom Beeckman
Faculty of Sciences, Department of Plant Biotechnology and Bioinformatics
Ghent University

Prof. Dr. Frank Van Breusegem
Faculty of Sciences, Department of Plant Biotechnology and Bioinformatics
Ghent University

Increasing crop productivity to sustain the growing world population has become a key objective of plant biotechnology. The final size of plant organs is controlled by a complex regulatory network of molecular players governed by different plant hormones. Extensive research on the genetic model organism *Arabidopsis thaliana* has led to the identification of many genes that improve growth when overexpressed or mutated. As such, plants with enhanced root growth due to overexpression of *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*) genes, and plants with increased leaf growth due to overexpression of the transcriptional regulators *ANGUSTIFOLIA 3* (*AN3*) and *GROWTH REGULATING FACTOR 5* (*GRF5*) were subject of ongoing research in the 'Systems Biology of Yield' group. Although the physiological aspect of the phenotypes of these transgenic plants has been described, little is known on the molecular basis of the phenotypes. Therefore, we set out to elaborately characterize these transgenic lines to identify novel mechanisms and genes that control Arabidopsis leaf size.

A large body of this work focusses on a detailed analysis of leaf growth, for which a firm understanding of the current knowledge is required. By means of introduction, a short overview of the evolution of photosynthetic leaves as major energy providers for the plant is first presented in **Chapter 1,** in the context of changing atmospheric CO_2 concentrations. Next, **Chapter 1** aims to give an overview of shoot development comprising meristem function and lateral organ formation, with emphasis on the molecular components that are involved. Because the phytohormone cytokinin gained a prominent role during this research, the published literature on its role during shoot development is reviewed extensively. This revealed that although ample information is available on the molecular players that control shoot apical meristem function, little is known on the genes that convey cytokinin responses in a developing leaf. Furthermore, sequence homology hints at a possible association of AN3 with epigenetic regulation of gene expression during initial leaf growth and therefore, **Chapter 1** concludes with an introduction on chromatin modifications. Similar to cytokinin regulation of leaf development, the epigenetic regulation of leaf development by chromatin remodeling complexes at the molecular level is far from being elucidated, leaving room for further research.

Stacking distinct phenotypic traits has gained interest in the field of plant biotechnology, and in **Chapter 2** our goal was to explore the feasibility of enhanced biomass production by combining enhanced root growth with enhanced shoot growth. Thereto, *CKX3* overexpressing plants were crossed with four different transgenic lines showing increased leaf size, and the effects on root and leaf growth were analyzed. Since transgenes were combined that belong to a diversity of functional classes, with this strategy we also aimed to characterize crosstalk between the different pathways in which these genes function.

Two cellular processes that contribute to the determination of final leaf size are cell proliferation and cell expansion. The remaining research chapters focus on the detailed characterization of three transcriptional regulators that stimulate leaf growth.

Overexpression of *GRF5* promotes cell division during early leaf development. Another interesting aspect of the phenotype however, is the darker green color of the leaves compared to wild-type leaves. In order to gain more insight into the nature of this phenotype, the chloroplasts and photosynthetic parameters of *GRF5* overexpressing leaves were studied in more detail in **Chapter 3**. Since alterations in chloroplast number or structure can affect nitrogen assimilation, an important biotechnological trait, the tolerance to growth on medium depleted of nitrogen was examined. Both cell division and chloroplast development are known to be stimulated by cytokinins, which suggests cross talk between GRF5 and cytokining signaling pathways, which was also investigated in **Chapter 3**.

Like GRF5, AN3 was shown to stimulate leaf cell division, but the molecular mechanisms acting upstream and downstream of AN3 are largely unknown. By inducible activation of AN3 in proliferating leaves followed by transcript profiling and qRT-PCR, we intended to extend the molecular network that regulates cell division downstream of AN3 in **Chapter 4**. Furthermore, AN3 was proposed to act in complex with GRF5, but how they interact genetically and how this transcription coactivator/transcription factor complex regulates gene expression is not known. To tackle these questions, rosette growth and target gene expression were analyzed in transgenic plants with modified levels of both *AN3* and *GRF5*. In addition, tandem affinity purification (TAP) was used to identify protein complexes that associate with AN3.

Another line of research in the department hinted at a putative role during leaf growth for *CYTOKININ RESPONSE FACTOR 3* (*CRF3*), which encodes a transcription factor likely involved in cytokinin signaling. In **Chapter 5**, we set out to characterize rosette growth of plants overexpressing *CRF3* to confirm its suggested role in the regulation of leaf size. A detailed phenotyping was performed by the specialized techniques optimized in the research group, in order to determine the cellular nature of the increased leaf area.

Finally, in **Chapter 6** I attempt to integrate the results from the different research chapters, which will contribute to a deeper understanding of the genetic network that regulates leaf growth. We not only expect to extend the network with new knowledge on existing molecular players and with the identification of new players, but also to shed more light on the relationship between them. This will aid in identifying candidate genes for genetic engineering to improve crop productivity.

ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
bHLH	Basic-helix-loop-helix
BL	Brassinolide
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
Chl	Chlorophyll
CoIP	Co-immunoprecipitation
Col-0	Columbia-0
CRY	Cryptochrome
cZ	Cis-zeatin
DAS	Days after stratification
D-box	Destruction box
DEX	Dexamethasone
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
FC	Fold change
Fd	Ferredoxin
FDR	False discovery rate
FM	Floral meristem
GA	Gibberellic acid
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
H	Histone
IM	Inflorescence meristem
iP	N^6-(Δ^2-isopentenyl)adenine
IYG	Intrinsic yield gene
miR	MicroRNA
mRNA	Messenger RNA
MS	Mass spectrometry
NADP	Nicotinamide adenine dinucleotide phosphate
PcG	Polycomb-group
Pchlide	Protochlorophyllide
PHY	Phytochrome
PPR	Pentatricopeptide repeat
PS	Photosystem
QC	Quiescent centre
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RAM	Root apical meristem

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SAM	Shoot apical meristem
SD	Standard deviation
SE	Standard error
TAP	Tandem affinity purification
tasiRNA	Trans-acting short interfering RNA
TCS	Two component output sensor
T-DNA	Transfer DNA
TrxG	Trithorax-group
tZ	Trans-zeatin
UTR	Untranslated region
WT	Wild type

1

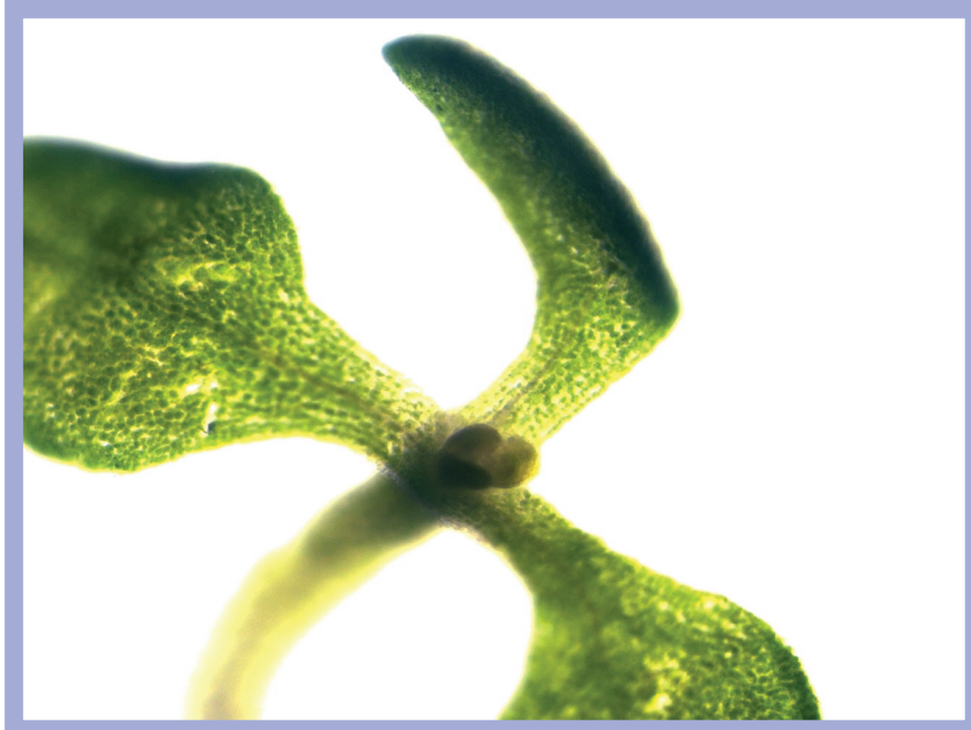


Image: Eight-day-old Arabidopsis Col-0 seedling containing three cotyledons and three first leaves.

Chapter 1: Introduction

THE IMPACT OF PLANT DEVELOPMENT ON EARTH'S EVOLUTION

Plants convert light energy from the sun into chemical energy by carbon assimilation during the process of photosynthesis. They gained the ability to perform photoautotrophic growth about 1500 million years ago (MYA), when the primary endosymbiosis event occurred, i.e. the uptake of plastids from cyanobacteria by eukaryotic organisms (Yoon et al., 2004). This event gave rise to photosynthetic algae and plants that were built up initially of axial stems lacking actual leaves. The subsequent development of megaphylls, the true leaf blades in vascular plants, around 360 MYA at the end of the Devonian era, coincided with a decline in atmospheric CO_2 because of its use for photosynthesis (Fig. 1, A) (Beerling, 2005; Berner, 2006; Royer, 2006; Leakey and Lau, 2012). As a consequence, earth's climate and ecosystem changed dramatically, making vertebrate life possible while accelerating terrestrial animal evolution.

As atmospheric CO_2 declined, stomatal density increased, leading to higher transpiration rates that allowed to increase photosynthesis without the risk of overheating (Fig. 1, B) (Beerling et al., 2001; Royer et al., 2001; Franks and Beerling, 2009; Leakey and Lau, 2012). Whereas at first the decreasing atmospheric CO_2 concentration was the driver of plant evolution, competition for light and space became the driving force that led to increased plant stature and leaf growth (Knoll and Niklas, 1987; Osborne et al., 2004). Larger plants also require enhanced root systems for stability and increased uptake of nutrients and water (Raven and Edwards, 2001).

A second period with decreasing CO_2 levels that created selective pressure for plant evolution, started 100 MYA and proceeds until the present day (Fig. 1, A) (Knoll and Niklas, 1987; Berner, 2006; Royer, 2006). It was preceded by an increase in vein density that started 150 MYA and putatively led to a higher hydraulic and stomatal conductance and density, resulting in higher CO_2 fixation (Fig. 1, B) (Royer et al., 2001; Franks and Beerling, 2009; Brodribb and Feild, 2010; Leakey and Lau, 2012). Also during this period of low CO_2, C4 photosynthesis in grasses and CAM (crassulacean acid metabolism) photosynthesis in orchids and cacti developed (Edwards and Smith, 2010)

(Fig. 1, B). These mechanisms concentrate CO_2 to maximize water use efficiency, a strategy to better cope with challenging environments.

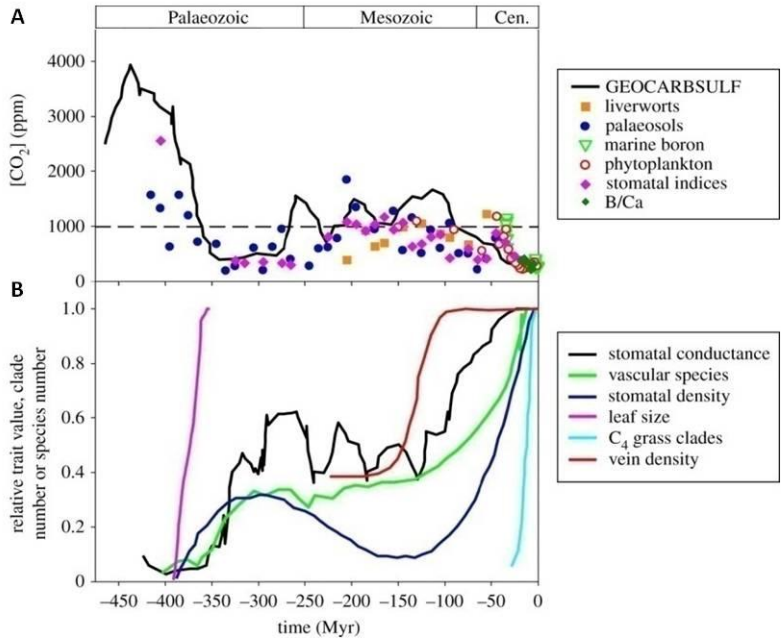


Figure 1. CO_2 concentration and plant evolution.

Comparative time courses over most of the Phanerozoic of: A, estimated atmospheric [CO_2] predicted from a geochemical model of the carbon cycle (GEOCARBSULF) and multiple proxies of [CO_2] (stomatal indices and isotope analysis of liverworts, palaeosols, marine boron, phytoplankton and B/Ca), with a dashed line at 1000 ppm indicating the atmospheric [CO_2] above which photosynthesis is saturated in most modern plants; B, estimated maximum stomatal conductance, estimated vascular species richness, stomatal density, Devonian and Carboniferous leaf size, C_4 grass clade richness and angiosperm vein density, all of which are expressed relative to the maximum value in the individual records of each parameter from the cited studies. Adapted from Leakey and Lau (2012), and references therein.

Today, rising CO_2 levels since the industrial revolution might influence plant growth, as declining levels did in the Paleozoic era 400 MYA and more recently since 100 MYA. As plant photosynthesis greatly affects cycling of nutrients, water and carbon, altering plant growth can have a major impact on the exchange of energy between the atmosphere and the land and hence on the amount of food, feed, fuel and fibers provided for terrestrial life on earth. However, until now, no substantial evidence is supplied for altered plant evolution under contemporary increases in atmospheric CO_2, although higher CO_2 levels

do greatly affect plant physiology (Leakey and Lau, 2012). Biotechnology holds nevertheless great promise to improve photosynthetic efficiency under current and higher future CO_2 levels to improve plant productivity (Zhu et al., 2010).

SHOOT DEVELOPMENT AND THE ROLE OF CYTOKININS

Plant development occurs largely post germination, from two populations of stem cells: the root apical meristem (RAM) and the shoot apical meristem (SAM), that give rise to the root and the shoot, respectively, and that need to be maintained during the plant's life. After cotyledon opening, leaves are initiated at the flanks of the SAM as rod-like primordia that develop into mature leaves with flat lamina. Depending on the environmental conditions, the SAM stops producing leaves at a certain moment and becomes an inflorescence meristem (IM) that generates the inflorescence and flowers, finally giving rise to seeds.

As sessile organisms of which the leaves serve as ultimate energy source for the plant, shoot development is tightly regulated but exhibits at the same time tremendous plasticity to adapt to changing environmental conditions. This is largely accomplished by the action of several phytohormones that integrate external stimuli with endogenous responses. Phytohormones act as local and long-distance signaling molecules that together regulate virtually all aspects of plant development. Kinetin was the first cytokinin to be isolated by Skoog and Miller (Miller et al., 1955a; Miller et al., 1955b), as the compound that promoted cell division, or cytokinesis, of plant tissue cultures together with auxin, the earliest discovered phytohormone (Went, 1928). High cytokinin/auxin levels promoted differentiation of tobacco callus into shoot tissue, while low cytokinin/auxin levels favor root development (Skoog and Miller, 1957), a key discovery for biotechnology, because of its application for plant regeneration after transformation.

During the past 15 to 20 years, extensive research on the model organism *Arabidopsis thaliana* (Arabidopsis) contributed enormously to our understanding of the cytokinin response pathway and its function during shoot growth on the physiological and molecular level.

Cytokinin metabolism and signaling

Besides a function in regeneration of shoot tissue from callus, cytokinins are well known for their positive role in seed germination, leaf expansion and photosynthesis while negatively affecting apical dominance and senescence (Mok, 1994). Evidence for the necessity of cytokinin function for Arabidopsis rosette growth accumulated over the past ten years with the analysis of overexpressors or mutants in components of cytokinin metabolism and signaling pathways.

Cytokinins are N^6-substituted adenine derivatives of which trans-zeatin (tZ), cis-zeatin (cZ) and N^6-(Δ^2-isopentenyl)adenine (iP) are the main naturally occurring species, biologically active as free bases (Mok and Mok, 2001). Their biosynthesis is accomplished by the combined action of ISOPENTENYLTRANSFERASES (IPTs) (Kakimoto, 2001; Takei et al., 2001a; Miyawaki et al., 2004; Miyawaki et al., 2006), CYTOCHROME P450 735A (CYP735A) hydroxylases (Takei et al., 2004), and the cytokinin-activating LONELY GUY (LOG) phosphoribohydrolases (Kurakawa et al., 2007; Kuroha et al., 2009; Tokunaga et al., 2012). Cytokinins are synthesized at various sites throughout the plant, where they can be activated and act as autocrine or paracrine signaling molecules. In addition, they are loaded as precursors into the vascular system to function as long-distance messengers, with tZ riboside predominantly transported acropetally via the transpiration stream while iP ribosides and ribotides are abundant in the phloem, suggesting a role in systemic and/or basipetal signaling (Beveridge et al., 1997; Takei et al., 2001b; Corbesier et al., 2003; Takei et al., 2004; Hirose et al., 2008; Matsumoto-Kitano et al., 2008; Kudo et al., 2010). Besides biosynthesis, activation and translocation, inactivation by glucose-conjugation (Mok, 1994; Mok and Mok, 2001), and degradation by CYTOKININ OXIDASES/DEHYDROGENASES (CKXs) (Bilyeu et al., 2001; Werner et al., 2003; Kowalska et al., 2010) contribute to the differential spatial and temporal distribution of the active cytokinin species.

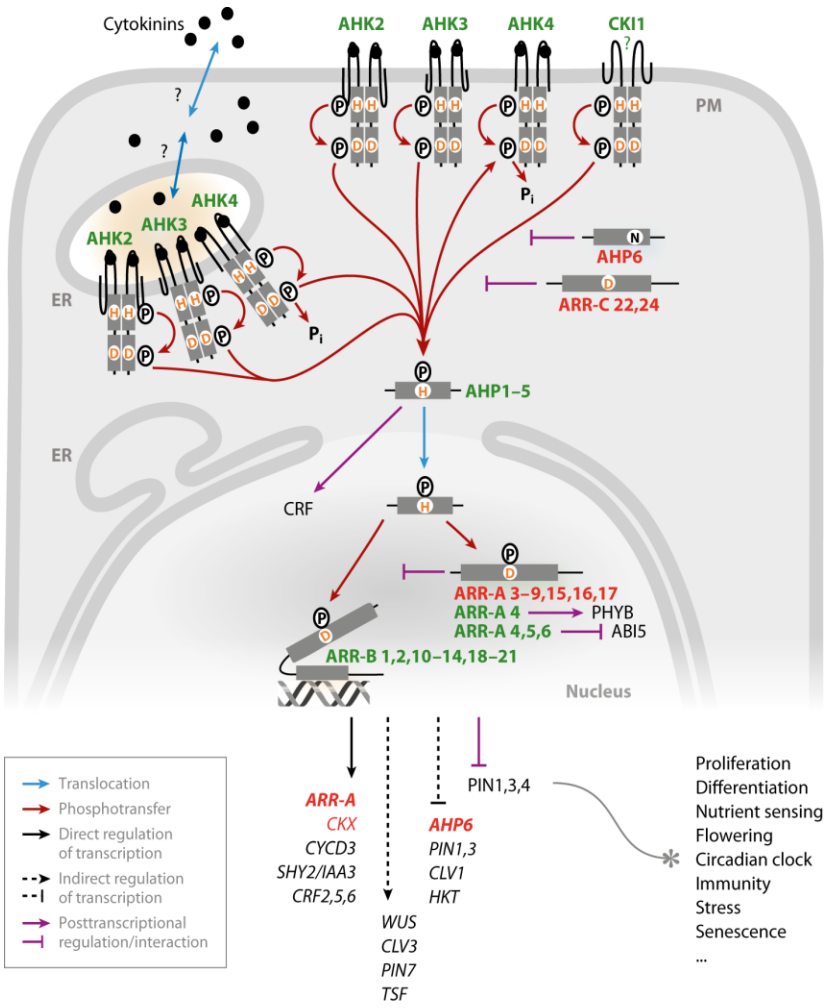
Cytokinins are perceived by the transmembrane CHASE (cyclases/histidine kinases associated sensory extracellular) domain of three hybrid ARABIDOPSIS HISTIDINE KINASES, AHK2, AHK3 and AHK4/CRE1/WOL (Fig. 2) (Mahonen et al., 2000; Inoue et al., 2001; Suzuki et al., 2001a; Ueguchi et al., 2001; Yamada et al., 2001; Heyl et al.,

2007). They function partially redundantly to initiate cytokinin signaling, since a major role has been appointed for AHK4 in the root, while AKH2 and AHK3 are more prominently involved in cytokinin perception in the shoot (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Stolz et al., 2011).

Upon cytokinin perception, a Histidine-to-Aspartate (His-to-Asp) two-component signaling mechanism is initiated by the autophosphorylation of the sensor domain of the receptor, followed by phosphotransfer to the carboxy-terminal receiver domain (Fig. 2) (Hwang and Sheen, 2001; Caesar et al., 2011; Wulfetange et al., 2011; Hwang et al., 2012). Subsequently, the phosphoryl group is transferred to ARABIDOPSIS HIS PHOSPHOTRANSFER proteins (AHPs), five of which are present in Arabidopsis (AHP1-5) (Miyata et al., 1998; Suzuki et al., 1998; Suzuki et al., 2000). They interact aspecifically with the receptors and function redundantly in transduction of the phosphosignal from the cytoplasm to a conserved Asp residue of 11 B-type ARABIDOPSIS RESPONSE REGULATORS (ARR1, 2, 10-14, 18-21) in the nucleus (Imamura et al., 1999; Suzuki et al., 2001b; Hutchison et al., 2006; Dortay et al., 2008).

Phosphorylation of the B-type ARRs results in transcription of the primary cytokinin targets, including the A-type *ARRs* (Fig. 2) (Sakai et al., 1998; Lohrmann et al., 1999; Hwang and Sheen, 2001; Sakai et al., 2001; Mason et al., 2005; Kim et al., 2006; Taniguchi et al., 2007; Brenner et al., 2012; Hwang et al., 2012). Mutant analysis and transcript profiling revealed that B-type ARRs, in particular ARR1, ARR10 and ARR12, are responsible for the majority of cytokinin-activated responses during vegetative plant development (Argyros et al., 2008; Heyl et al., 2008; Ishida et al., 2008).

The 10 A-type ARRs (ARR3-9, 15-17) contain only short carboxy-terminal extensions most likely lacking transcription factor properties, and function as negative feedback regulators of the two component pathway (Brandstatter and Kieber, 1998; Imamura et al., 1999; D'Agostino et al., 2000; Kiba et al., 2003; To et al., 2004; Lee et al., 2007). Like B-type ARRs, they contain a conserved Asp residue in their amino-terminal receiver domain that requires phosphorylation for proper function, because it putatively affects protein binding (To et al., 2007; Lee et al., 2008a). Because their interaction was shown with AHPs but not B-type ARRs, it is postulated that they attenuate cytokinin signaling at the level of the AHPs (Fig. 2) (Dortay et al., 2006).



Hwang I, et al. 2012.
Annu. Rev. Plant Biol. 63:353–80

Figure 2. The core cytokinin signaling circuitry.

Cytokinin signaling through AHKs, AHPs and ARRs in a model cell. Conserved His and Asp residues, which accept a phosphoryl group (P), are indicated by orange H and D letters, respectively. Boldface indicates core signaling components, green indicates positive regulators of cytokinin signaling, and red indicates negative regulators. Selected connections to other signals and genes are indicated. Additional abbreviations: ER, endoplasmic reticulum; PM, plasma membrane. From (Hwang et al., 2012).

Another branch increasing the complexity of cytokinin signaling is delineated by the CYTOKININ RESPONSE FACTOR (CRF) family, comprising 12 members (Rashotte et al., 2006; Rashotte and Goertzen, 2010). *CRF2*, *CRF5* and *CRF6* were shown to be primary cytokinin response genes and CRF1-6 proteins relocalize to the nucleus upon cytokinin application (Rashotte et al., 2006; Brenner et al., 2012). This movement was demonstrated for CRF2 to be dependent on phosphorylation and on the presence of the AHKs and AHPs, but not the ARRs (Rashotte et al., 2006). In addition, CRF1-8 were shown to homo- and heterodimerize and interact with the AHPs (Cutcliffe et al., 2011). Moreover, CRFs regulate transcription of a large portion of cytokinin-response genes, many of them which are also differentially regulated by B-type ARRs, placing the CRF signaling module as a parallel pathway branching from the AHPs, that regulates a significant part of cytokinin responsive transcription (Fig. 2) (Rashotte et al., 2006).

In conclusion, the multiple branches of the two component signaling pathway combined with diverse layers of feedback regulation and post-transcriptional modifications, result in a fine-tuned balance in cytokinin signaling for optimal plant development.

That cytokinins are essential for Arabidopsis shoot development is illustrated for instance by overexpression of *CKX* genes, leading to enhanced cytokinin breakdown, resulted in a decrease in SAM size, delayed leaf formation, diminished leaf cell production, reduced vascular development and hampered reproductive development (Werner et al., 2003). These characteristic developmental defects are, together with an increased root architecture, collectively typified as the cytokinin deficiency syndrome. Furthermore, higher order mutations in the biosynthetic *ipt* and *log* genes and abolished cytokinin perception in the *ahk2/ah3/ahk4* triple mutant, similarly resulted in dwarfed growth with a smaller SAM and less leaves with decreased vein density (Higuchi et al., 2004; Nishimura et al., 2004; Miyawaki et al., 2006; Riefler et al., 2006; Tokunaga et al., 2012). Likewise, the *ahp* quintuple mutant displayed reduced shoot growth, although rather less severe (Hutchison et al., 2006), while the rosettes of the B-type *arr1/arr10/arr12* triple mutant and plants ectopically expressing *ARR1* as a dominant negative repressor (*35S:ARR1-SRDX*) were also strongly reduced in size (Argyros et al., 2008; Heyl et al., 2008; Ishida et al., 2008).

Shoot apical meristem establishment

Leaves, stems and flowers arise from the shoot apical meristem. To assure a consecutive formation of lateral organs, a population of cells in the central zone of the SAM needs to divide continuously. This is accomplished by a tight regulation on the genetic level, governed by the interplay of cytokinins with other hormones.

Key to this regulation is a positive feedback loop, comprising CLAVATA3 (CLV3), a peptide secreted from the central zone and WUSHEL (WUS), a homeodomain transcription factor expressed in cells of the organizing centre underneath the central zone (Fig. 3) (Mayer et al., 1998; Fletcher et al., 1999). WUS positively affects stem cell activity, but promotes expression of *CLV3*, a negative regulator, in cells of the central zone. CLV3 in turn acts, via its receptor CLV1, to confine *WUS* expression in the underlying organizing centre, together providing a mechanism for stem cell homeostasis in the SAM (Clark et al., 1997; Brand et al., 2000; Schoof et al., 2000; Ogawa et al., 2008; Wolters and Jurgens, 2009; Barton, 2010; Perales and Reddy, 2012).

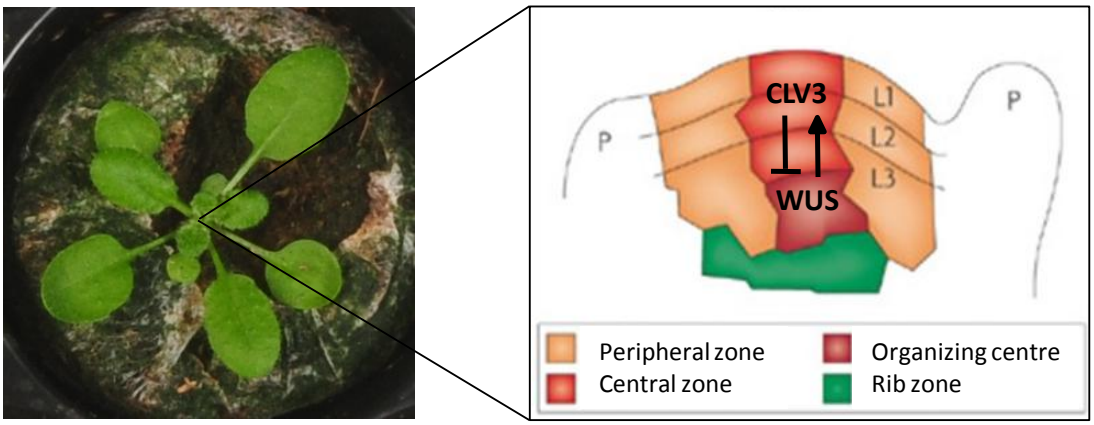


Figure 3. The shoot apical meristem (SAM).

The location of the SAM is shown in a 22-day-old soil-grown Arabidopsis plant on the left. The structure of the SAM is shown on the right with the different types of cells in the meristem illustrated by the different colors. L1, L2 and L3, meristem layers; P, primordium or primordium initiation site. The positive feed-back loop comprising CLV3 and WUS is indicated. Adapted from (Wolters and Jurgens, 2009).

In addition, the class-I *KNOTTED 1-LIKE HOMEOBOX* (*KNOX*) gene family, consisting of *SHOOT MERISTEMLESS* (*STM*); *BREVIPEDICELLUS* (*BP*)/*KNAT1*, *KNAT2* and *KNAT6*, are more broadly expressed in the SAM compared to *WUS* and *CLV3* and have an important role in SAM maintenance (Lincoln et al., 1994; Long et al., 1996; Pautot et al., 2001; Belles-Boix et al., 2006). Whereas STM action is essential in the SAM, BP and KNAT6 were shown to function partially redundant with STM (Long et al., 1996; Byrne et al., 2002; Belles-Boix et al., 2006).

Cytokinin action in the SAM

The earliest sign of cytokinin signaling that directs shoot development, was reported during embryogenesis. GFP fluorescence from a TWO COMPONENT output SENSOR (*TCS:GFP*), containing consensus B-type ARR-binding motifs in the promoter (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003), could be observed in the founder cell of the shoot stem cell population at the heart stage (Müller and Sheen, 2008). Cytokinins are important for the maintenance of the SAM, and the molecular players are well described. The initial observation that *stm* mutation could be rescued by cytokinin treatment (Long et al., 1996), can now be explained by the induction of *IPT7* by STM (Jasinski et al., 2005; Yanai et al., 2005). Elevated IPT levels in turn have the potential to activate *STM* and *KNAT1* expression (Fig. 4) (Rupp et al., 1999).

WUS represses transcription of A-type *ARRs*, *ARR5*, *ARR6*, *ARR7* and *ARR15*, by direct promoter binding as demonstrated for *ARR7*, thereby establishing a positive feedback loop reinforcing cytokinin signaling in the organizing centre (Leibfried et al., 2005). *ARR7* and *ARR15* mRNAs accumulate in the central zone where they putatively reduce cytokinin signaling. Interestingly their expression overlaps with the *CLV3* transcript domain and it was suggested that they are indispensible for proper CLV3 expression (Zhao et al., 2010). Conversely, cytokinins upregulate the expression of *WUS* via AHK2 and AHK4, both independently and through repression of *CLV1* expression. Furthermore *TCS:GFP* expression peaks in the *WUS* expression domain, concomitant with the *AHK4* expression pattern (Fig. 4) (Lindsay et al., 2006; Gordon et al., 2009). In parallel, elevated *WUS* levels were reported in the *ckx3ckx5* double mutant and *LOG* genes were

shown to be expressed in the central zone, indicating that also cytokinin activation and degradation contribute to the fine-tuning of cytokinin activity in the SAM (Fig. 4) (Yadav et al., 2009; Bartrina et al., 2011).

Thus, cytokinins are controlling and being controlled by master regulators in the SAM, resulting in multiple feed forward mechanisms that ascertain a constant stem cell pool. Furthermore, there is cross talk with other hormones. Synergism with auxin signaling for cell division maintenance was provided by AUXIN RESPONSE FACTOR5 (ARF5)/MONOPTEROS (MP), that directly represses *ARR7* and *ARR15* to exclude them from the peripheral zone (Fig. 4) (Zhao et al., 2010). GIBBERELLIC ACID (GA) activity on the other hand needs to be suppressed for proper meristem function, which is illustrated by the transcriptional repression of the biosynthetic gene *GA 20-OXIDASE1* (*GA20ox1*) by STM and by the antagonizing induction of the catabolic gene *GA2ox2* by cytokinin signaling (Hay et al., 2002; Jasinski et al., 2005).

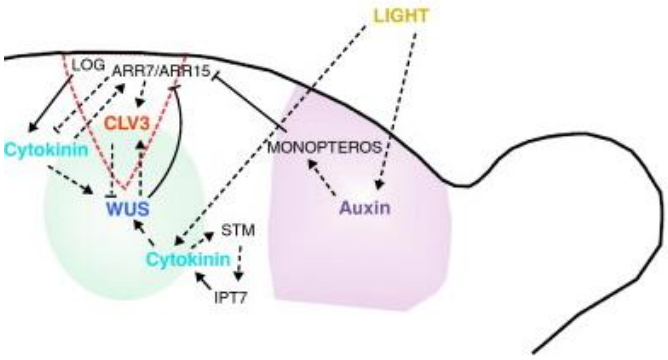


Figure 4. Schematic representation of a gene regulatory network linking cytokinin and auxin action in SAM maintenance.

The SAM domain that responds to auxin is colored in soft purple whereas the SAM domain that preferentially perceives cytokinin is shown in soft blue. Light signaling also contributes to the local modulation of auxin and cytokinin metabolism. Continuous lines represent direct connections, whereas broken lines represent indirect links. Modified from (Perales and Reddy, 2012).

Light is a necessary signal for SAM function and organ initiation. It was shown in tomato that in the absence of light, the local auxin maxima at incipient primordia are lost due to loss of proper PIN1 distribution. This could not be rescued by local exogenous auxin application alone, but also needed cytokinin in the dark, indicating that cytokinins are

also necessary for lateral organ initiation in the dark (Yoshida et al., 2011). Moreover, the *amp1* mutant, that has an increased cytokinin content, recues the *mp* mutant phenotype characterized by the absence of leaves (Vidaurre et al., 2007). Thus, although cytokinin and auxin stimulate opposing processes, cytokinin promotes stem cell fate, while auxin promotes differentiation, they cannot accomplish their function without each other.

Leaf primordium initiation

The new cells arising from the SAM are pushed to the peripheral zone, where they will commit to initiate lateral organ primordia (Fig. 5). In order for the founder cells to differentiate, *KNOX* gene transcription needs to be repressed at the site of primordium formation (Fig. 5). This is accomplished in part by a complex containing ASYMMETRIC LEAVES 1 (AS1) and AS2, that represses *BP*, *KNAT2* and *KNAT6* gene expression, most likely by direct promoter binding (Byrne et al., 2000; Semiarti et al., 2001; Guo et al., 2008). STM in turn was shown to downregulate *AS1* expression (Byrne et al., 2000; Byrne et al., 2002). Furthermore, CUP-SHAPED COTYLEDON (CUC) transcription factors are necessary for lateral organ boundary establishment and SAM organization, and they were shown to act upstream of STM (Aida et al., 1997; Takada et al., 2001; Hibara et al., 2003; Vroemen et al., 2003; Hibara et al., 2006).

Another prerequisite for lateral organ initiation is the formation of a local auxin maximum (Fig. 5), by polar auxin transport mediated by the auxin efflux carrier PINFORMED1 (PIN1) (Reinhardt et al., 2003; Wiśniewska et al., 2006). Auxin is thought to contribute to *KNOX* downregulation, resulting simultaneously in a local rise in GA levels, due to the lack of repression of *GA20ox1* by STM, at the site of primordium formation (Hay et al., 2002; Heisler et al., 2005). Auxin maxima also prevent the formation of new primordia in close proximity, a mechanism that assures a correct spacing between subsequent primordia and hence determines phyllotaxis (Jonsson et al., 2006). Stimulation of PIN1 localization and transcription by PLETHORA/AINTEGUMENTA-LIKE (PLT/AIL) transcription factors was shown to be involved in phyllotaxis determination (Fig. 5), since the plt3/plt5/plt7 triple mutant had decussate instead of spiral phyllotaxis concomitant with misregulation of PIN1 (Prasad et

al., 2011). *PLT* genes form together with *AINTEGUMENTA* (*ANT*) a subfamily comprising eight members within the AP2/ERF family of transcription factors, with partially overlapping but also distinct roles in SAM maintenance and lateral organ outgrowth and development (Nole-Wilson et al., 2005; Krizek, 2009, 2011; Prasad et al., 2011; Mudunkothge and Krizek, 2012).

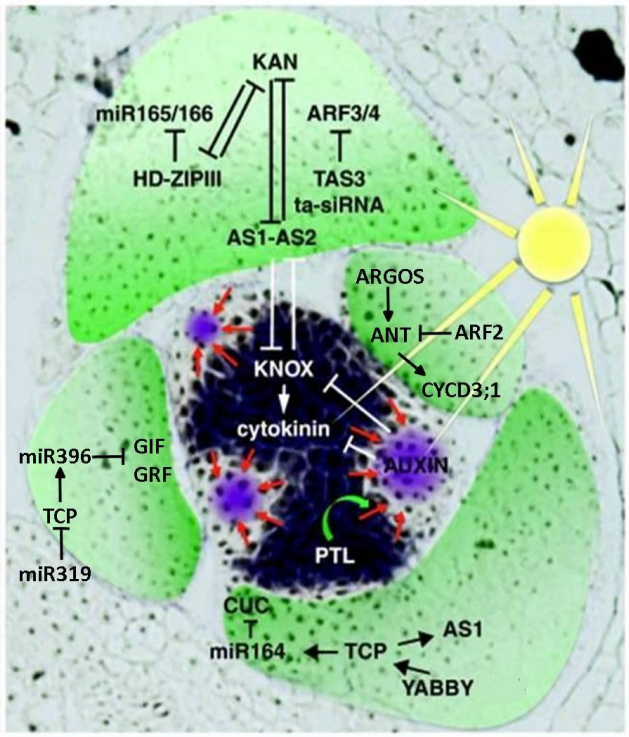


Figure 5. Genetic interactions involved in leaf initiation and development.
SAM function is maintained through *KNOX* gene activation of cytokinin. Polar auxin transport by PIN1 (red arrows) leads to auxin maxima (purple), which establishes sites of organ initiation. Auxin in initiating leaf primordia represses *KNOX* genes and cytokinin signaling. Light (yellow) is required for SAM function and organ initiation. See text for further details on the molecular players in the leaves. Adapted from Byrne (2012).

Leaf blade formation

After the establishment of the primordium, appearing as a rod-like structure off the flank of the SAM, the leaves need to develop three distinct axes: proximo-distal, medio-lateral, and dorso-ventral. Several antagonizing molecular players have been identified that mediate proper polar growth. Whereas for the dorso-ventral axis for instance, the class III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III) proteins PHAVOLUTA (PHV), REVOLUTA (REV) and PHABULOSA (PHB) determine adaxial fate, KANADI (KAN) and YABBY (YAB) proteins define the abaxial domain. HD-ZIP III and KAN mutually repress each other's expression. In addition, *HD-ZIP III* mRNAs are confined to the

adaxial side because they are targeted by microRNA165/166 (miR165/166), expressed in the abaxial side. In addition, the AS1/AS2 complex promotes *HD-ZIP III* expression and simultaneously reduces *KAN*, *YAB* and miR165/166 expression, while KAN directly suppresses *AS2*. Moreover, miR165/166 and AUXIN RESPONSE FACTOR3/ETTIN (ARF3/ETT) that stimulate abaxialisation, are counteracted by trans-acting short interfering RNAs (tasiRNAs) expressed in the adaxial domain (Fig. 5) (reviewed by Moon and Hake, 2011; Byrne, 2012).

Other molecular players influence leaf blade outgrowth along the leaf length axis, by influencing the transition from cell proliferation to cell expansion, thereby controlling the progression of the mitotic arrest front. For example, ANT, GROWTH REGULATING FACTORS (GRFs), GRF INTERACTING FACTORS (GIFs), KLUH, and DA1 all are important for maintenance of cell proliferation capacity in a growing leaf (Mizukami and Fischer, 2000; Kim et al., 2003; Kim and Lee, 2006; Anastasiou et al., 2007; Li et al., 2008; Lee et al., 2009; Gonzalez et al., 2012). ANT stimulates *CYCD3;1* expression in developing leaves, thus sustaining the mitotic cell cycle (Mizukami and Fischer, 2000). ANT itself is a downstream target of AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE (ARGOS), whose gain and loss of function increases or decreases leaf cell number, respectively, similar to gain and loss of ANT function (Mizukami and Fischer, 2000; Hu et al., 2003). The regulation by auxin is corroborated by the finding that *ANT* expression is negatively regulated by AUXIN RESPONSE FACTOR 2 (ARF2), a general repressor of cell proliferation in aerial organs (Fig. 5) (Schruff et al., 2006).

Overexpression of *GIF1/ANGUSTIFOLIA 3* (*AN3*), *GIF2* and *GIF3* enhances leaf growth by increased cell division, while *an3*, *gif2* and *gif3* mutations cause a synergistic reduction in cell number (Kim and Kende, 2004; Horiguchi et al., 2005; Lee et al., 2009). Similar observations were made in *GRF1*, *GRF2* and *GRF5* overexpressing plants or single and higher order *grf* mutants (Kim et al., 2003; Horiguchi et al., 2005; Kim and Lee, 2006; Gonzalez et al., 2010). Combinations of *gif* and *grf* mutations synergistically decrease leaf size and the GIF transcriptional coactivators interact with the GRF transcription factors, demonstrating that they function together in regulating gene transcription to promote cell division (Kim and Kende, 2004; Horiguchi et al., 2005; Kim and Lee, 2006). Seven *GRFs* (*GRF1-4*, *GRF7-9*) are targeted by *miR396* that is expressed

at the tip of young leaf primordia where cell expansion starts and accumulates during leaf growth to restrict *GRF* expression to the basal part of the leaf (Jones-Rhoades and Bartel, 2004; Liu et al., 2009; Rodriguez et al., 2010; Wang et al., 2011). *MiR396* expression is in turn stimulated by TEOSINTE BRANCHED1/CYCLOIDEA/PCF 4 (TCP4). Elevated levels of TCP4 as well as *miR396* overexpression itself reduce the transcription of all *GRFs* and *AN3* resulting in smaller leaves with less cells (Fig. 5) (Liu et al., 2009; Rodriguez et al., 2010; Wang et al., 2011).

The class II TCPs, containing TCP2, 3, 4, 5, 10, 13, 17 and 24, are basic-helix-loop-helix (bHLH) transcription factors, generally repressing growth by inhibiting cell proliferation. TCP2, 3, 4, 10 and 24 contain a target site for miR319, and downregulation of their mRNA levels by ectopic expression of *miR319*, referred to as *jaw-D*, resulted in crinkled leaves due to excessive cell proliferation at the leaf margins, similar to the phenotype of the multiple *tcp* knockouts (Fig. 5) (Palatnik et al., 2003; Efroni et al., 2008; Schommer et al., 2008; Koyama et al., 2010).

Increasing complexity

The different pathways that control development along the different axes do not just work independently, since several interactions have been identified. As such, class II TCPs were in addition shown to repress expression of boundary specific genes, by the direct upregulation of yet another *miRNA*, *miR164*, that targets *CUC* mRNA for degradation (Fig. 5), a mechanism analogous to repression of the *GRFs* by upregulation of *miR396* (Laufs et al., 2004; Koyama et al., 2007; Koyama et al., 2010). Furthermore, *AS1*, *INDOLE-3-ACETIC ACID3/SHORT HYPOCOTYL2* (IAA/SHY2) and a *SMALL AUXIN UP RNA* (*SAUR*) gene were identified as directly transcriptionally induced by TCP3 and to cooperatively suppress *CUC* expression (Koyama et al., 2010). Complexity is further increased by the finding that class II TCPs interact with AS2 and bind the promoters of *BP* and *KNAT2* to inhibit their expression and stem cell fate in incipient leaf primordia (Li et al., 2012a). Moreover, *TCP* expression itself is promoted by YABBY function, which simultaneously leads to repression of class I *KNOX* genes (Fig. 5) (Kumaran et al.,

2002; Sarojam et al., 2010). Taken together, class II TCPs contribute to restrict gene expression for correct morphogenesis, stimulating leaf differentiation.

The fact that leaf formation is extremely tightly regulated is demonstrated again by the observation that reduced *GRF* levels, by overexpression of *miR396*, as well as loss of *an3* function enhanced the adaxial/abaxial defects of *as1* and/or *as2* (Horiguchi et al., 2011; Wang et al., 2011). Likewise, *ant* mutation enhances polarity defects together with *yabby* mutations (Nole-Wilson and Krizek, 2006). This indicates that active cell proliferation in the leaf primordium is necessary for adaxial/abaxial polarity establishment.

When cell proliferation ceases, cell differentiation is initiated and the increase of leaf blade size is mainly driven by cell expansion. This requires besides the TCPs and other molecular players, EXPANSINS (EXPs) that mediate cell wall loosening (Cho and Cosgrove, 2000; Cosgrove, 2005; Sampedro and Cosgrove, 2005) and SAUR proteins that most likely regulate auxin transport (Spartz et al., 2012). Finally, prolonged division of meristemoids during the cell expansion phase contributes to final leaf size, and is for example negatively controlled by PEAPOD proteins (White, 2006; Gonzalez et al., 2012).

Cytokinin action during leaf blade development

Cytokinin signaling is indispensable for leaf blade expansion, judging from the reduction in size of the leaves, due to less cells and diminished vascular development in mutants with reduced cytokinin content or signaling (Werner et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Hutchison et al., 2006; Miyawaki et al., 2006; Riefler et al., 2006; Argyros et al., 2008; Heyl et al., 2008; Ishida et al., 2008; Tokunaga et al., 2012). It was proposed that cytokinins stimulate procambial cell proliferation and maintenance, but suppress protoxylem differentiation, thereby antagonizing auxin action, at least in the root. The components of the cytokinin signaling pathway that are involved are beginning to be uncovered (reviewed by Hwang et al., 2012).

Despite the extensive knowledge on cytokinin action in the shoot meristem, little molecular information is available on how cytokinin promotes leaf cell division. Direct regulation of cell cycle genes could be a possibility. *CYCD3* genes regulate the extent of

cell proliferation and their expression in dividing cells of the SAM and leaf primordia is enhanced by cytokinins and essential for cytokinin function (Riou-Khamlichi et al., 1999; Dewitte et al., 2007). Recently, a role in promoting cytokinin effects was suggested for two class I TCPs, generally postulated to promote cell proliferation (Li et al., 2005; Martin-Trillo and Cubas, 2010; Steiner et al., 2012). *TCP14* and *TCP15* are expressed in young developing leaves and overexpression of *TCP14* enhances *CYCB1;2* marker gene expression synergistically with cytokinin treatment (Kieffer et al., 2011; Li et al., 2012b; Steiner et al., 2012). In addition, a detailed analysis of *ANT:CKX3* leaf growth, where cytokinin degradation was directed to the leaf primordia by expression of *CKX3* under control of the *ANT* promoter, revealed that cytokinins specifically act to delay the exit from the mitotic cell cycle and simultaneously delay the start of differentiation (Holst et al., 2011). This is in agreement with the potential involvement of CYC proteins, but a direct link is still missing. Furthermore, *ANT:CKX3* leaves had decreased cell numbers, but larger cells. This well documented compensated cell enlargement was however more pronounced than in *35S:CKX3* leaves, although the latter have a stronger reduction in cell numbers, suggesting that cytokinins might also play a role during cell expansion (Tsukaya, 2008; Holst et al., 2011). Furthermore, one might not forget that cytokinins promote photosynthesis and sink strength, processes that are likely to influence the extent of leaf growth (Wareing et al., 1968; Werner et al., 2008). In conclusion, there is room for extensive future research to uncover the direct molecular interactions with the cytokinin signaling pathway in a developing leaf.

Floral transition and flower development

The switch to reproductive development can be triggered by different external and internal signals. Long day conditions for instance, lead to the stabilization of the CONSTANS (CO) transcription factor that directly activates FLOWERING LOCUS T (FT) in the phloem, which moves from the leaves to the SAM to form a putative complex with the bZIP transcription factor FD (Suarez-Lopez et al., 2001; Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Imaizumi, 2010). SUPPRESSOR OF CONSTANS 1 (SOC1), another transcription

factor, is subsequently activated and in turn directly regulates expression of *LEAFY* (*LFY*) to initiate flowering (Samach et al., 2000; Lee et al., 2008b). The members of the photoperiod pathway are also targets of the vernalization and autonomous pathways, that mediate the downregulation of the floral repressor *FLOWERING LOCUS C* (*FLC*), which leads to the upregulation of *FT* and *SOC1* (Koornneef et al., 1998; Hepworth et al., 2002; Moon et al., 2003; Helliwell et al., 2006; Searle et al., 2006). In addition, the GA pathway and a recently identified autonomous pathway controlled by plant age, converge on *SOC1* and *LFY* (Blazquez et al., 1998; Moon et al., 2003; Yamaguchi et al., 2005; Eriksson et al., 2006). Although these floral integrators play a predominant role in the five pathways that initiate flowering, numerous other players were described to be part of the complex gene regulatory network that regulates floral transition (reviewed in Srikanth and Schmid, 2011).

Upon bolting the SAM is transformed into the IM, that initiates numerous floral meristems (FM) which grow out to become flowers. Several genes that are involved in leaf initiation are also important for flower development. FMs are formed in the axils of rudimentary bracts, that protrude from the IM and are associated with the expression of *ANT*, *STM* and *LFY* (Long and Barton, 2000; Grandjean et al., 2004; Kwiatkowska, 2006). *CUC2* expression on the other hand defines the boundary between the IM and the FM (Breuil-Broyer et al., 2004; Laufs et al., 2004). Expression of *LFY* is necessary and sufficient for floral organ identity establishment and defines together with *APETALA1* (*AP1*) expression the first stages of flower development. The flower meristem gives rise to the four floral whorls including sepals, petals, stamens and carpels, that are specified by the *ABC* homeotic genes. In short, the *A*-genes, *AP1* and *AP2*, define sepals, the *A*-genes together with the *B*-genes, *AP3* and *PISTILLATA* (*PI*), define petals, the *B*-genes plus the *C*-gene, *AGAMOUS* (*AG*), specify stamens and the *C*-gene alone specifies carpels. Furthermore, *ABC* gene expression needs to be accompanied by the expression of four *SEPALATA* (*SEP*) genes to be sufficient for correct floral whorl identity (reviewed in Alvarez-Buylla et al., 2010). Loss of all three *abc* and four *sep* functions results in the conversion of flowers into leaf-like structures, while ectopic expression of *ABC* and *SEP* genes convert leaves into floral organs, indicating that flowers are of the same origin as

leaves (Fig. 6) (Bowman et al., 1991; Honma and Goto, 2001; Pelaz et al., 2001; Ditta et al., 2004).

The requirement for cytokinins during flowering is less clear and not well documented, but recently shown to be rather stimulating than inhibiting (D'Aloia et al., 2011).

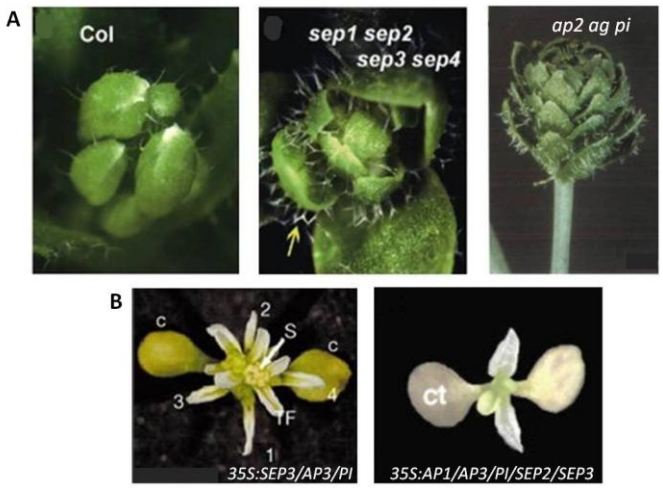


Figure 6. A, Phenotypes of quadruple *sep* mutant flowers and triple mutant flowers lacking *ABC* function. B, Phenotypes of rosettes overexpressing *SEP* and *ABC* genes. C or ct: cotyledons. S: stamens. Numbers show the order of leaf development (modified from Bowman et al., 1991; Honma and Goto, 2001; Pelaz et al., 2001; Ditta et al., 2004)

Photomorphogenesis

As light serves as the major energy source for plants, it influences plant growth throughout its lifecycle. When a seed germinates in the soil devoid of light, the hypocotyl of the seedling will elongate rapidly to reach the light before seed reserves are depleted. The immature cotyledons are folded and squeezed tightly together while the hypocotyl forms an apical hook to protect the SAM from damage by the soil during this process. When the seedling reaches the light, the hypocotyl stops elongating, the apical hook and cotyledons open, etioplasts differentiate into chloroplasts and the apical meristems are activated. The characteristic etiolated growth of seedlings in the dark is termed skotomorphogenesis, and contrasts with photomorphogenesis, when seeds germinate in the light (reviewed by Arsovski et al., 2012). In addition, light affects circadian rhythms and flowering time, and the direction of growth, for example by avoiding shade (Alvarez-Buylla et al., 2010; Imaizumi, 2010; Casal, 2012).

Light is perceived by four classes of photoreceptors, the phytochromes, cryptochromes, phototropins and UVB photoreceptors, and key downstream components have been characterized that simultaneously serve as important integrators of light and hormonal pathways (Fig. 7) (Wang and Deng, 2004; Lau and Deng, 2010; Yu et al., 2010; Chaves et al., 2011; Rizzini et al., 2011). These include CONSTITUTIVE PHOTOMORPHOGENIC 1/DE-ETIOLATED/FUSCA1 (COP1/DET/FUS1), an E3 ubiquitin ligase, LONG HYPOCOTYL 5 (HY5), a basic leucine zipper (bZIP) transcription factor and PHYTOCHROME-INTERACTING FACTOR 1/PIF3-LIKE 5 (PIF1/PIL5), PIF3, PIF4 and PIF5, four members of a bHLH subfamily of transcription factors (Deng et al., 1991; Oyama et al., 1997; Leivar et al., 2008; Leivar and Quail, 2011). In the dark, COP1 directs HY5 for degradation by the 26S proteasome while the PIFs are stabilized (Osterlund et al., 2000; Leivar et al., 2008). Moreover, the stability of PIF proteins is enhanced by COP1 and the PIFs stimulate COP1 to mark phytochrome B (PHYB) for degradation, a positive feedback loop assuring repression of photomorphogenesis in the dark (Bauer et al., 2004; Jang et al., 2010). Upon light perception by the phytochromes, COP1 is repressed leading to accumulation of HY5 while the PIFs are in turn degraded by the proteasome upon phosphorylation by the activated phytochromes, resulting in transcription that promotes photomorphogenesis and inhibits skotomorphogenesis (Fig. 7) (Osterlund et al., 2000; Al-Sady et al., 2006; Shen et al., 2007; Leivar et al., 2008; Lau and Deng, 2010).

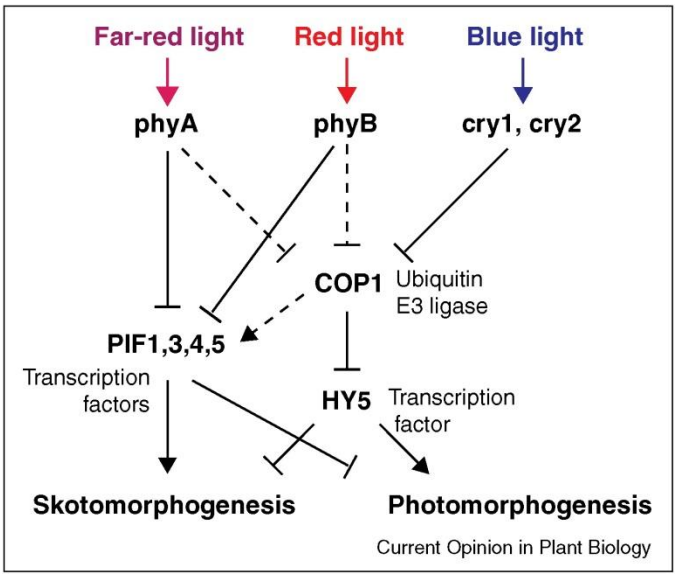


Figure 7. A simplified model of the light signaling pathway.
Phytochromes (phyA and phyB) and cryptochromes (cry1 and cry2) sense different qualities of light. Under light, COP1 is repressed by phytochromes and cryptochromes and phytochromes directly interact with PIFs, resulting in PIFs' degradation. Between two proteins, solid lines indicate a direct effect, while dotted lines represent an indirect regulation. Taken from Lau and Deng (2010).

Cytokinin action during photomorphogenesis

Exogenous cytokinin application is known to evoke light-grown phenotypes in the dark, like inhibition of hypocotyl elongation, cotyledon opening, leaf formation and chloroplast development. Loss of *cop1/det/fas1* function resulted in reduced sensitivity to cytokinin in shoot tissue formation from callus (Chory et al., 1994). Evidence on the molecular level was provided by the fact that cytokinins enhance the stability of HY5, most likely through inhibition of COP1, thereby promoting photomorphogenesis (Vandenbussche et al., 2007).

GA on the other hand is widely known to promote skotomorphogenesis, by removing the growth suppressing DELLAs that otherwise inhibit PIF3 and PIF4 function and by reducing HY5 protein levels (de Lucas et al., 2008; Feng et al., 2008). In contrast, GA promotes seed germination together with light, as is illustrated at the molecular level by transcriptional activation of GA catabolic genes and DELLAs by PIF1, that repress GA signaling in the dark (Oh et al., 2006; Oh et al., 2007). Interestingly, *CRF1*, *CRF2* and *CRF3* were shown to be direct targets that are downregulated by PIF1, suggesting that cytokinin signaling also needs to be repressed to inhibit seed germination in the dark (Oh et al., 2009).

An important discovery was the light-dependent direct interaction of ARR4 with PHYB, resulting in stabilization of the active form of PHYB (Sweere et al., 2001). It was postulated that the cytokinin-activated two component phosphorelay regulates ARR4 to affect PHYB dynamics, resulting in the integration of light signaling and cytokinin signaling. Light sensitizes the seedlings for cytokinin, while cytokinin reduces the hypersensitivity of seedlings to light (Mira-Rodado et al., 2007). Furthermore, ARR4 was shown to function together with PHYB, and redundantly with ARR3, in the regulation of light input into the circadian clock (Hanano et al., 2006; Salome et al., 2006; Zheng et al., 2006), indicating that A-type ARRs not only give negative feedback on cytokinin signaling, but also positively mediate cytokinin responses.

In addition, a reduction in the cytokinin response induced by shade, has been associated with decreased Chla/b levels and decreased photosynthetic capacity (Boonman et al., 2007; Boonman et al., 2009). Moreover, *CKX5* is induced within 1h in response to shade

in a PIF-dependent manner, and also *CKX6* is upregulated by shade (Carabelli et al., 2007; Leivar et al., 2012). This reinforces the finding that cytokinins work together with light, via photoreceptor interaction and through the two main light signaling pathways, to balance external signals with internal requirements.

CHROMATIN REMODELING

During the plant's life cycle, subsequent developmental programs need to be run through which involves the activation of certain gene sets, while repressing others. Transcription factors, transcriptional coactivators and corepressors, the general transcription machinery, and chromatin modifications cooperate to ensure that the correct genes are expressed in the required amount during an exactly defined timeframe (Maston et al., 2006). Remodeling of the chromatin is crucial to establish and maintain gene expression patterns. Not only do chromatin remodelers play a key role during cell differentiation in promoting cell fate, but they are also required during cell proliferation to stimulate meristematic gene expression, implicating that the epigenetic state needs to be conserved throughout several rounds of mitosis and DNA replication (Jarillo et al., 2009).

Chromatin remodeling mechanisms are conserved among plants, animals and yeast, and much of our knowledge in plants is based on the homology with animal and yeast chromatin remodeling proteins (Jerzmanowski, 2007; Kwon and Wagner, 2007). However, whereas in animals the majority of the developmental fates and concomitant epigenetic states are established early during embryogenesis, plants develop largely post-embryonic and exhibit a plasticity to respond to changing environmental stimuli. Therefore, plants often contain families of functionally redundant chromatin remodeling proteins, not always resulting in lethal single mutant phenotypes and allowing for a more detailed functional analysis (Jarillo et al., 2009).

Chromatin remodeling is accomplished by two main types of modifications: covalent modifications of the histones and the DNA, and the ATP-driven alterations that change the interactions between the histones and the DNA. Both modifications are carried out in

general by large multi-protein complexes and their activities are frequently interlinked (Wagner, 2003; Jarillo et al., 2009).

Covalent chromatin modifications

A plethora of covalent modifications of histone residues has been reported and include acetylation, methylation, phosphorylation, sumoylation and ubiquitination, and is collectively referred to as the epigenome (Kouzarides, 2007; Roudier et al., 2009). These covalent modifications affect nucleosome positioning and compaction resulting in differences in the accessibility of the DNA to transcription factors, coactivators or corepressors and the RNA polymerase machinery. Epigenomic mapping has contributed to a general understanding of the role of the covalent histone marks (Roudier et al., 2009). For instance, trimethylation of lysine 4 of HISTONE3 (H3K4me3) has been associated with active transcription, whereas H3K27me3 marks are frequently found in transcriptionally repressed chromatin. Likewise, hyperacetylation by HISTONE ACETYL TRANSFERASES (HATs) is a general mark for activation of transcription, while hypoacetylation mediated by HISTONE DEACETYLASES (HDACs) is generally associated with transcriptional repression (Jarillo et al., 2009; Roudier et al., 2009; Berr et al., 2011). Besides histones, also the DNA can be covalently modified, with as main example the hypermethylation of heterochromatic DNA that is frequently linked with stable inheritable silencing (Vaillant and Paszkowski, 2007).

During plant development, the epigenome is continuously changing concomitant with changes in cell fate. In the centre of the SAM and IM for instance, the cells need to maintain meristematic identity, while in the peripheral zone, cells differentiate to initiate lateral organ primordia such as leaves and flowers. Chromatin modifications are necessary to keep meristematic genes activated and differentiation genes repressed in the central zone, while doing the opposite at the site of incipient primordia. These opposing effects are generally accomplished by two groups of genes, the Polycomb-Group (PcG) and the Trithorax-group (TrxG), that promote repressive and active states of gene expression, respectively, in part by covalently modifying histones (Pien and Grossniklaus, 2007).

As such, the PcG complex, POLYCOMB REPRESSIVE COMPLEX 1 (PRC1) is responsible for the maintenance of the repressive chromatin state that has been established by PRC2 which evokes H3K27 methylation at target loci (Pien and Grossniklaus, 2007). PRC2 is a multisubunit complex, consisting of a HISTONE METHYLTRANSFERASE (HMTase), in Arabidopsis encoded by *MEDEA* (*MEA*), *CURLY LEAF* (*CLF*) or *SWINGER* (*SWN*), and three other core components based on homology with *Drosophila*, of which two are also encoded by gene families in Arabidopsis. These include *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) and *EMBRYONIC FLOWER 2* (*EMF2*), amongst others (Kohler and Villar, 2008). By mutational analysis, CLF and FIE were shown to be involved in silencing of the meristem identity genes *STM*, *KNAT1*, *KNAT2* and *KNAT6* in leaf primordia (Goodrich et al., 1997; Katz et al., 2004). Furthermore, CLF was demonstrated to bind the *STM* locus and to function partially redundant with SWN in its H3K27 methylation (Makarevich et al., 2006; Schubert et al., 2006). The floral homeotic gene *AG* is also a target of CLF activity and probably SWN activity in complex with EMF2 and FIE, resulting in repression of *AG* during leaf growth (Goodrich et al., 1997; Chanvivattana et al., 2004; Katz et al., 2004; Schubert et al., 2006). In addition, FIE was suggested to contribute to silencing of *AP3* and *PI* (Kinoshita et al., 2001).

In *Drosophila*, the four-component PRC1 complex binds trimethylated H3K27 marks to maintain stable silencing by ubiquitination of H2 (Zheng and Chen, 2011). Although no orthologs could be identified in Arabidopsis, the presence of functional homologs has been demonstrated. LIKE HETEROCHROMATIN PROTEIN 1/TERMINAL FLOWER 2 (LHP1/TFL2) would be responsible for association with the H3K27me3 throughout the genome (Turck et al., 2007; Zhang et al., 2007), while RING1A and RING1B and BMI1A and BMI1B interact with LHP and are potent candidates to confer the ubiquitination activity (Xu and Shen, 2008; Bratzel et al., 2010; Zheng and Chen, 2011). Consistent with a function succeeding that of PRC2, PRC1 appears to be involved in silencing of the flower homeotic genes *AG*, *AP3* and *PI* (See also Fig. 9) (Nakahigashi et al., 2005; Germann et al., 2006; Turck et al., 2007). Moreover, the putative involvement of both complexes in repression of *FLC* in response to vernalization, and repression of

FT during vegetative growth, illustrates the importance of PcG genes in repression of master regulatory genes (Turck et al., 2007; Jarillo and Pineiro, 2011).

During the switch from one developmental stage to the next, PcG repression often needs to be eliminated, e.g. for floral organ initiation (Pien and Grossniklaus, 2007). This is accompanied by methylation of H3K4 by members of the TrxG proteins, like TRITHORAX 1 (ATX1), the loss of function of which results in downregulation of *AG*, *AP3* and *PI* expression (See also Fig. 9) (Alvarez-Venegas et al., 2003; Saleh et al., 2007).

ATP-dependent chromatin remodeling

The second type of chromatin modification involves ATP-hydrolysis to alter the DNA accessibility for the transcription machinery. This is executed by multi-subunit complexes that are formed around a central ATPase that provides the energy for processes such as nucleosome movement, conformational changes or removal of histones and nucleosomes, resulting in both closed or open chromatin states and thus repression or activation of target gene transcription (Fig. 8) (Kwon and Wagner, 2007; Hargreaves and Crabtree, 2011). Four evolutionary conserved classes of complexes can be distinguished, based on the type of ATPase that is present: SWITCH/SUCROSE NONFERMENTING (SWI/SNF), CHROMODOMAIN-HELICASE-DNA-BINDING (CHD), IMITATION SWITCH (ISWI), and INO80, of which the first three play a role in the control of gene expression during key developmental processes (Jerzmanowski, 2007; Kwon and Wagner, 2007; Hargreaves and Crabtree, 2011).

Of particular interest to the research presented here are the SWI/SNF complexes. They consist of at least five different conserved subunits, of which four are encoded by gene families in Arabidopsis. As such, four ATPases were described, BRAHMA (BRM), SPLAYED (SYD), CHR12 and CHR23; four SWI3 proteins (SWI3A-D); two SWI/SNF ASSOCIATED PROTEINs 73 (SWP73A and B); two ACTIN RELATED PROTEINS (ARP4 and 7) predicted to belong to SWI/SNF complexes; and one protein termed BUSHY (BSH) (Fig. 8) (The Chromatin Database, www.chromdb.org; Meagher et al., 2005; Jerzmanowski, 2007; Kwon and Wagner, 2007). Homologs of the subunits were

initially discovered in yeast by the inability of loss of function mutants to switch mating type and to ferment sucrose (Neigeborn and Carlson, 1984; Stern et al., 1984; Breeden and Nasmyth, 1987). Likewise, knock-out mutations or knock-down of genes encoding plant SWI/SNF subunits have dramatic effects on plant growth, characterized by severely reduced growth, a dwarfed stature, altered flowering time and impaired flower development (Brzeski et al., 1999; Wagner and Meyerowitz, 2002; Zhou et al., 2003; Farrona et al., 2004; Kandasamy et al., 2005a; Sarnowski et al., 2005; Hurtado et al., 2006; Crane and Gelvin, 2007; Archacki et al., 2009). Moreover, loss of certain subunits causes embryo lethality, together illustrating the importance of SWI/SNF chromatin remodeling throughout the plant's life cycle (Kandasamy et al., 2005b; Sarnowski et al., 2005).

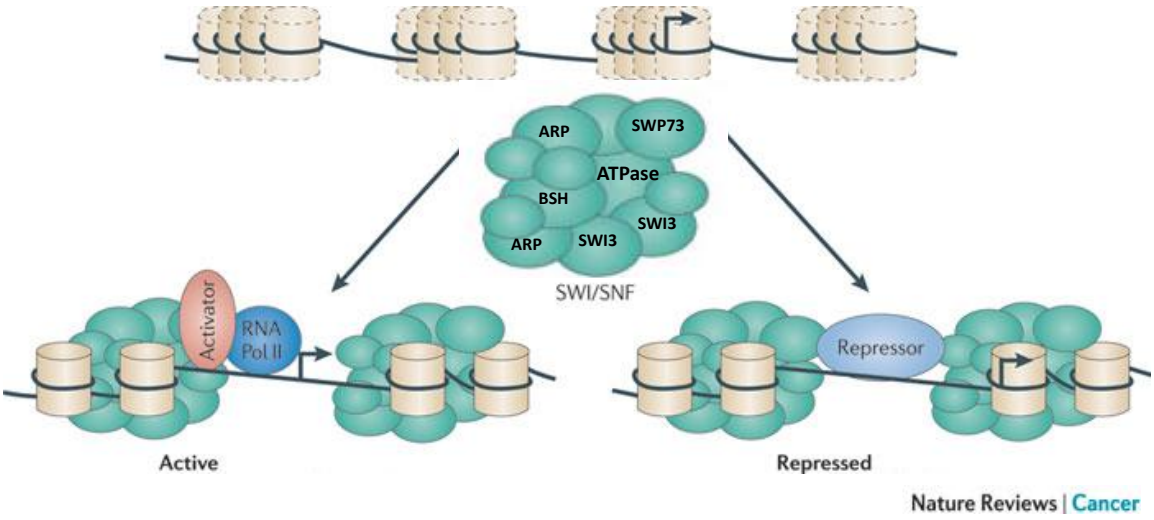


Figure 8. Mode of action of SWI/SNF complexes.

Model of a SWI/SNF complex with the putative Arabidopsis conserved subunits indicated by their names. Empty circles represent associated subunits. In contrast to silent chromatin regions (top of the figure), at active genes that are rich in SWI/SNF binding (bottom left of the figure) the transcription start site (indicated by an arrow) is flanked by precisely positioned nucleosomes, thus providing unobstructed access to a nucleosome-depleted region that contains transcription factor binding sites. SWI/SNF complexes also contribute to the dynamic silencing of targets that are required for lineage-specific differentiation and that facilitate the binding of repressors (bottom right of the figure). Adapted from Wilson and Roberts (2011).

Genetic analyses have broadened our understanding of how SWI/SNF complexes affect several plant developmental processes at the molecular level. During embryogenesis, the formation of cotyledon boundaries is controlled by three functionally redundant *CUC* genes (*CUC1*, *2* and *3*), all positively regulated by BRM, while transcription of only *CUC2* is controlled by SYD (Kwon et al., 2006). Leaves develop asymmetrically and adaxial fate is in part promoted by *FILAMENTOUS FLOWER* (*FIL*), a *YABBY* gene family member whose expression is reduced by mutation of *SYD* (Eshed et al., 2004). SYD is a direct positive regulator of *WUS* transcription and hence co-responsible for the maintenance of the SAM (Kwon et al., 2005), whereas the interaction of SYD with BRCA1-ASSOCIATED RING DOMAIN 1 (BARD1), could lead to the inhibition of its remodeling activity outside the *WUS* expression domain (Han et al., 2008). On the other hand, levels of the class-I *KNOX* gene *KNAT6*, which is involved in SAM maintenance, cotyledon boundary formation, and leaf and inflorescence development, are under negative control of SYD in leaves (Su et al., 2006). Different pathways influence the transition to flowering and BRM involvement in the photoperiodic and autonomous pathways has been shown by repression of *FT*, *CO* and *SOC1* as well as *FLC* (Farrona et al., 2004; Farrona et al., 2011). Also SYD is involved in floral transition by downregulation of *FT* expression and LFY activity (Wagner and Meyerowitz, 2002; Su et al., 2006). Furthermore, because expression of homeotic genes is downregulated in mutant plants, SYD, BRM, SWI3C and SWI3D activate homeotic gene expression (Wagner and Meyerowitz, 2002; Sarnowski et al., 2005; Hurtado et al., 2006; Su et al., 2006; Farrona et al., 2007). Finally, genes encoding seed storage proteins (SSP) need to be highly expressed during seed maturation but strongly repressed in vegetative tissue, and the latter is accomplished by a SWI/SNF complex including BRM, SWI3C and BSH (Tang et al., 2008). Thus, key developmental switches during plant growth are controlled by SWI/SNF chromatin remodeling, and complexes consisting of different paralogous components can regulate transcription of overlapping but also unique target genes (Bezhani et al., 2007; Wu et al., 2012).

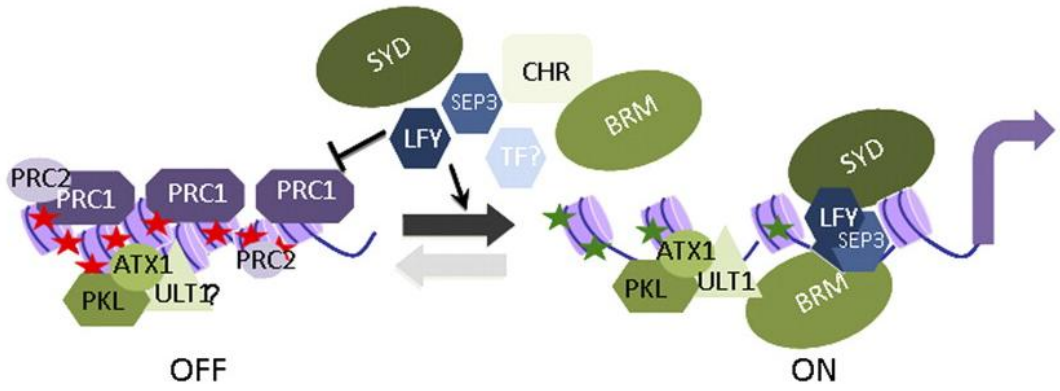


Figure 9. Model for *AP3* or *AG* induction and reversal of polycomb repression.
In seedlings and leaves as well as in nonexpressing tissues of the flower (OFF state), PRC1 and PRC2 and high levels of H3K27me3 (red stars) are present. Two TrxG proteins, PKL and ATX1, are also thought to be present at this time, whereas occupancy of a third TrxG-like putative coactivator ULTRAPETALA (ULT1) is not known (Carles and Fletcher, 2009). Floral homeotic gene activation (ON state) requires LFY, SEP3, and possibly additional transcription factors (TFs), which recruit SYD and BRM to the regulatory regions of *AP3* and *AG*. ULT1 and other chromatin factors (CHR) may also be recruited at this time. In addition, LFY directly represses expression of the presumptive PRC1 complex (Winter et al., 2011; Zheng and Chen, 2011). The combined activities result in loss of PRC2, H3K27me3, and PRC1 and lead to accumulation of H3K4me3 (green stars). Taken from Wu et al. (2012).

Furthermore, SYD and BRM were recently proposed to have TrxG-like activity, by overcoming PcG repression mediated by PRC1 and PRC2 at *AP3* and *AG* loci during the activation of floral homeotic gene expression (Fig. 9) (Wu et al., 2012). However, the above described findings show that SWI/SNF complexes also act to repress transcription of master regulators that are also repressed by PcG proteins. Similar observations were made for the ATPase PICKLE (PKL), belonging to the CHD class of ATP-dependent chromatin remodeling complexes. PKL represses *KNOX* gene transcription in newly formed leaf primordia in association with AS1 (Ori et al., 2000). In addition, PKL was recently shown to negatively affect photosynthetic gene expression concomitant with inhibiting cytokinin-induced callus growth and greening, and its repressive activity was suggested to be associated with HDAC-activity and H3K27 trimethylation (Furuta et al., 2011; Zhang et al., 2012). In contrast, PKL has also been proposed to act as a TrxG protein in Arabidopsis antagonizing PcG repression at the *AP3* and *AG* loci (Fig. 9) (Aichinger et al., 2009; Kohler and Aichinger, 2010). This illustrates that chromatin

remodeling ATPases most likely cannot be unequivocally labeled as TrxG proteins, but rather are important activators and repressors of transcription that help balancing cellular meristematic fate with cell differentiation.

Besides the conserved subunits, the complexes are assembled of numerous associated proteins that affect the enzymatic activity of the complex, facilitate binding with other chromatin modifying enzymes, and guide the SWI/SNF complexes towards modified histones and specific DNA sequences (Hargreaves and Crabtree, 2011). These proteins are often transcriptional coactivators, corepressors or transcription factors, sometimes with a tissue specific expression pattern, that recruit SWI/SNF complexes for a correct spatiotemporal lineage specific differentiation. For example, the two transcription factors LFY and SEP3 physically interact with SYD and BRM to guide them to the promoter of *AP3* and *AG* resulting in transcription necessary for correct floral whorl formation (Fig. 9) (Wu et al., 2012). In addition, SWI3B was shown to interact with FCA, a regulator of *FLC* (Sarnowski et al., 2002), but to our knowledge, no other associated proteins have been identified in Arabidopsis. As such, SWI/SNF epigenetic control of gene expression in Arabidopsis is far from being elucidated.

REFERENCES

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science **309:** 1052-1056

Aichinger E, Villar CBR, Farrona S, Reyes JC, Hennig L, Kohler C (2009) CHD3 Proteins and Polycomb Group Proteins Antagonistically Determine Cell Identity in Arabidopsis. Plos Genetics **5**

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997) Genes involved in organ separation in Arabidopsis: An analysis of the cup-shaped cotyledon mutant. Plant Cell **9:** 841-857

Al-Sady B, Ni W, Kircher S, Schäfer E, Quail PH (2006) Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. Molecular Cell **23:** 439-446

Alvarez-Buylla ER, Benitez M, Corvera-Poire A, Chaos Cador A, de Folter S, Gamboa de Buen A, Garay-Arroyo A, Garcia-Ponce B, Jaimes-Miranda F, Perez-Ruiz RV, Pineyro-Nelson A, Sanchez-Corrales YE (2010) Flower development. Arabidopsis Book **8:** e0127

Alvarez-Venegas R, Pien S, Sadder M, Witmer X, Grossniklaus U, Avramova Z (2003) ATX-1, an Arabidopsis homolog of trithorax, activates flower homeotic genes. Current Biology **13:** 627-637

Anastasiou E, Kenz S, Gerstung M, MacLean D, Timmer J, Fleck C, Lenhard M (2007) Control of plant organ size by *KLUH/CYP78A5*-dependent intercellular signaling. Developmental Cell **13:** 843-856

Archacki R, Sarnowski TJ, Halibart-Puzio J, Brzeska K, Buszewicz D, Prymakowska-Bosak M, Koncz C, Jerzmanowski A (2009) Genetic analysis of functional redundancy of BRM ATPase and ATSWI3C subunits of Arabidopsis SWI/SNF chromatin remodelling complexes. Planta **229:** 1281-1292

Argyros RD, Mathews DE, Chiang YH, Palmer CM, Thibault DM, Etheridge N, Argyros DA, Mason MG, Kieber JJ, Schaller GE (2008) Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. Plant Cell **20:** 2102-2116

Arsovski AA, Galstyan A, Guseman JM, Nemhauser JL (2012) Photomorphogenesis. Arabidopsis Book **10:** e0147

Barton MK (2010) Twenty years on: The inner workings of the shoot apical meristem, a developmental dynamo. Developmental Biology **341:** 95-113

Bartrina I, Otto E, Strnad M, Werner T, Schmulling T (2011) Cytokinin Regulates the Activity of Reproductive Meristems, Flower Organ Size, Ovule Formation, and Thus Seed Yield in Arabidopsis thaliana. Plant Cell **23:** 69-80

Bauer D, Viczián A, Kircher S, Nobis T, Nitschke R, Kunkel T, Panigrahi KCS, Ádám E, Fejes E, Schäfer E, Nagy F (2004) Constitutive photomorphogenesis 1 and multiple photoreceptors control degradation of phytochrome interacting factor 3, a transcription factor required for light signaling in Arabidopsis. Plant Cell **16:** 1433-1445

Beerling DJ (2005) Leaf evolution: Gases, genes and geochemistry. Annals of Botany **96:** 345-352

Beerling DJ, Osborne CP, Chaloner WG (2001) Evolution of leaf-form in land plants linked to atmospheric CO2 decline in the Late Palaeozoic era. Nature **410:** 352-354

Belles-Boix E, Hamant O, Witiak SM, Morin H, Traas J, Pautot V (2006) KNAT6: an Arabidopsis homeobox gene involved in meristem activity and organ separation. Plant Cell **18:** 1900-1907

Berner RA (2006) GEOCARBSULF: A combined model for Phanerozoic atmospheric O2 and CO2. Geochimica Et Cosmochimica Acta **70:** 5653-5664

Berr A, Shafiq S, Shen WH (2011) Histone modifications in transcriptional activation during plant development. Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms **1809:** 567-576

Beveridge CA, Murfet IC, Kerhoas L, Sotta B, Miginiac E, Rameau C (1997) The shoot controls zeatin riboside export from pea roots. Evidence from the branching mutant rms4. Plant Journal **11:** 339-345

Bezhani S, Winter C, Hershman S, Wagner JD, Kennedy JF, Kwon CS, Pfluger J, Su YH, Wagner D (2007) Unique, shared, and redundant roles for the Arabidopsis SWI/SNF chromatin remodeling ATPases BRAHMA and SPLAYED. Plant Cell **19:** 403-416

Bilyeu KD, Cole JL, Laskey JG, Riekhof WR, Esparza TJ, Kramer MD, Morris RO (2001) Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol **125:** 378-386

Blazquez MA, Green R, Nilsson O, Sussman MR, Weigel D (1998) Gibberellins promote flowering of Arabidopsis by activating the LEAFY promoter. Plant Cell **10:** 791-800

Boonman A, Prinsen E, Gilmer F, Schurr U, Peeters AJM, Voesenek LACJ, Pons TL (2007) Cytokinin import rate as a signal for photosynthetic acclimation to canopy light gradients. Plant Physiol **143:** 1841-1852

Boonman A, Prinsen E, Voesenek LACJ, Pons TL (2009) Redundant roles of photoreceptors and cytokinins in regulating photosynthetic acclimation to canopy density. J Exp Bot **60:** 1179-1190

Bowman JL, Smyth DR, Meyerowitz EM (1991) Genetic Interactions among Floral Homeotic Genes of Arabidopsis. Development **112:** 1-20

Brand U, Fletcher JC, Hobe M, Meyerowitz EM, Simon R (2000) Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. Science **289:** 617-619

Brandstatter I, Kieber JJ (1998) Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. Plant Cell **10:** 1009-1019

Bratzel F, Lopez-Torrejon G, Koch M, Del Pozo JC, Calonje M (2010) Keeping Cell Identity in Arabidopsis Requires PRC1 RING-Finger Homologs that Catalyze H2A Monoubiquitination. Current Biology **20:** 1853-1859

Breeden L, Nasmyth K (1987) Cell-Cycle Control of the Yeast Ho Gene - Cis-Acting and Trans-Acting Regulators. Cell **48:** 389-397

Brenner WG, Ramireddy E, Heyl A, Schmulling T (2012) Gene regulation by cytokinin in Arabidopsis. Front Plant Sci **3:** 8

Breuil-Broyer S, Morel P, de Almeida-Engler J, Coustham V, Negrutiu I, Trehin C (2004) High-resolution boundary analysis during Arabidopsis thaliana flower development. Plant Journal **38:** 182-192

Brodribb TJ, Feild TS (2010) Leaf hydraulic evolution led a surge in leaf photosynthetic capacity during early angiosperm diversification. Ecology Letters **13:** 175-183

Brzeski J, Podstolski W, Olczak K, Jerzmanowski A (1999) Identification and analysis of the Arabidopsis thaliana BSH gene, a member of the SNF5 gene family. Nucleic Acids Research **27:** 2393-2399

Byrne ME (2012) Making leaves. Current Opinion in Plant Biology **15:** 24-30

Byrne ME, Barley R, Curtis M, Arroyo JM, Dunham M, Hudson A, Martienssen RA (2000) Asymmetric leaves1 mediates leaf patterning and stem cell function in Arabidopsis. Nature **408:** 967-971

Byrne ME, Simorowski J, Martienssen RA (2002) ASYMMETRIC LEAVES1 reveals knox gene redundancy in Arabidopsis. Development **129:** 1957-1965

Caesar K, Thamm AMK, Witthoft J, Elgass K, Huppenberger P, Grefen C, Horak J, Harter K (2011) Evidence for the localization of the Arabidopsis cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. J Exp Bot **62:** 5571-5580

Carabelli M, Possenti M, Sessa G, Ciolfi A, Sassi M, Morelli G, Ruberti I (2007) Canopy shade causes a rapid and transient arrest in leaf development through auxin-induced cytokinin oxidase activity. Genes & Development **21:** 1863-1868

Carles CC, Fletcher JC (2009) The SAND domain protein ULTRAPETALA1 acts as a trithorax group factor to regulate cell fate in plants. Genes & Development **23:** 2723-2728

Casal JJ (2012) Shade avoidance. Arabidopsis Book **10:** e0157

Chanvivattana Y, Bishopp A, Schubert D, Stock C, Moon YH, Sung ZR, Goodrich J (2004) Interaction of polycomb-group proteins controlling flowering in Arabidopsis. Development **131:** 5263-5276

Chaves I, Pokorny R, Byrdin M, Hoang N, Ritz T, Brettel K, Essen LO, van der Horst GTJ, Batschauer A, Ahmad M (2011) The Cryptochromes: Blue Light Photoreceptors in Plants and Animals. Annual Review of Plant Biology, Vol 62 **62:** 335-364

Cho H-T, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences of the United States of America **97:** 9783-9788

Chory J, Reinecke D, Sim S, Washburn T, Brenner M (1994) A role for cytokinins in de-etiolation in *Arabidopsis*: *det* mutants have an altered response to cytokinins. Plant Physiology **104:** 339-347

Clark SE, Williams RW, Meyerowitz EM (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell **89:** 575-585

Corbesier L, Prinsen E, Jacqmard A, Lejeune P, Van Onckelen H, Perilleux C, Bernier G (2003) Cytokinin levels in leaves, leaf exudate and shoot apical meristem of Arabidopsis thaliana during floral transition. J Exp Bot **54:** 2511-2517

Corbesier L, Vincent C, Jang SH, Fornara F, Fan QZ, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science **316:** 1030-1033

Cosgrove DJ (2005) Growth of the plant cell wall. Nature Reviews Molecular Cell Biology **6:** 850-861

Crane YM, Gelvin SB (2007) RNAi-mediated gene silencing reveals involvement of Arabidopsis chromatin-related genes in Agrobacterium-mediated root transformation. Proceedings of the National Academy of Sciences of the United States of America **104:** 15156-15161

Cutcliffe JW, Hellmann E, Heyl A, Rashotte AM (2011) CRFs form protein-protein interactions with each other and with members of the cytokinin signalling pathway in Arabidopsis via the CRF domain. J Exp Bot **62:** 4995-5002

D'Agostino IB, Deruere J, Kieber JJ (2000) Characterization of the response of the arabidopsis response regulator gene family to cytokinin. Plant Physiol **124:** 1706-1717

D'Aloia M, Bonhomme D, Bouche F, Tamseddak K, Ormenese S, Torti S, Coupland G, Perilleux C (2011) Cytokinin promotes flowering of Arabidopsis via transcriptional activation of the FT paralogue TSF. Plant Journal **65:** 972-979

de Lucas M, Davière J-M, Rodríguez-Falcón M, Pontin M, Iglesias-Pedraz JM, Lorrain S, Fankhauser C, Blázquez MA, Titarenko E, Prat S (2008) A molecular framework for light and gibberellin control of cell elongation. Nature **451:** 480-484

Deng XW, Caspar T, Quail PH (1991) Cop1 - a Regulatory Locus Involved in Light-Controlled Development and Gene-Expression in Arabidopsis. Genes & Development **5:** 1172-1182

Dewitte W, Scofield S, Alcasabas AA, Maughan SC, Menges M, Braun N, Collins C, Nieuwland J, Prinsen E, Sundaresan V, Murray JAH (2007) *Arabidopsis* CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proceedings of the National Academy of Sciences of the United States of America **104:** 14537-14542

Ditta G, Pinyopich A, Robles P, Pelaz S, Yanofsky MF (2004) The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Current Biology **14:** 1935-1940

Dortay H, Gruhn N, Pfeifer A, Schwerdtner M, Schmulling T, Heyl A (2008) Toward an interaction map of the two-component signaling pathway of Arabidopsis thaliana. Journal of Proteome Research **7:** 3649-3660

Dortay H, Mehnert N, Burkle L, Schmulling T, Heyl A (2006) Analysis of protein interactions within the cytokinin-signaling pathway of Arabidopsis thaliana. Febs Journal **273:** 4631-4644

Edwards EJ, Smith SA (2010) Phylogenetic analyses reveal the shady history of C-4 grasses. Proceedings of the National Academy of Sciences of the United States of America **107:** 2532-2537

Efroni I, Blum E, Goldshmidt A, Eshed Y (2008) A protracted and dynamic maturation schedule underlies *Arabidopsis* leaf development. Plant Cell **20:** 2293-2306

Eriksson S, Bohlenius H, Moritz T, Nilsson O (2006) GA(4) is the active gibberellin in the regulation of LEAFY transcription and Arabidopsis floral initiation. Plant Cell **18:** 2172-2181

Eshed Y, Izhaki A, Baum SF, Floyd SK, Bowman JL (2004) Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. Development **131:** 2997-3006

Farrona S, Hurtado L, Bowman JL, Reyes JC (2004) The Arabidopsis thaliana SNF2 homolog AtBRM controls shoot development and flowering. Development **131:** 4965-4975

Farrona S, Hurtado L, March-Diaz R, Schmitz RJ, Florencio FJ, Turck F, Amasino RM, Reyes JC (2011) Brahma Is Required for Proper Expression of the Floral Repressor FLC in Arabidopsis. Plos One **6**

Farrona S, Hurtado L, Reyes JC (2007) A nucleosome interaction module is required for normal function of Arabidopsis thaliana BRAHMA. Journal of Molecular Biology **373:** 240-250

Feng S, Martinez C, Gusmaroli G, Wang Y, Zhou J, Wang F, Chen L, Yu L, Iglesias-Pedraz JM, Kircher S, Schäfer E, Fu X, Fan L-M, Deng XW (2008) Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. Nature **451:** 475-479

Fletcher JC, Brand U, Running MP, Simon R, Meyerowitz EM (1999) Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. Science **283:** 1911-1914

Franks PJ, Beerling DJ (2009) CO2-forced evolution of plant gas exchange capacity and water-use efficiency over the Phanerozoic. Geobiology **7:** 227-236

Furuta K, Kubo M, Sano K, Demura T, Fukuda H, Liu YG, Shibata D, Kakimoto T (2011) The CKH2/PKL Chromatin Remodeling Factor Negatively Regulates Cytokinin Responses in Arabidopsis Calli. Plant and Cell Physiology **52:** 618-628

Germann S, Juul-Jensen T, Letarnec B, Gaudin V (2006) DamID, a new tool for studying plant chromatin profiling in vivo, and its use to identify putative LHP1 target loci. Plant Journal **48:** 153-163

Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhondt S, Van Daele T, De Milde L, Weigel D, Kamiya Y, Stitt M, Beemster GTS, Inzé D (2010) Increased leaf size: different means to an end. Plant Physiology **153:** 1261-1279

Gonzalez N, Vanhaeren H, Inzé D (2012) Leaf size control: complex coordination of cell division and expansion. Trends in Plant Science **in press**

Goodrich J, Puangsomlee P, Martin M, Long D, Meyerowitz EM, Coupland G (1997) A polycomb-group gene regulates homeotic gene expression in Arabidopsis. Nature **386:** 44-51

Gordon SP, Chickarmane VS, Ohno C, Meyerowitz EM (2009) Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. Proceedings of the National Academy of Sciences of the United States of America **106:** 16529-16534

Grandjean O, Vernoux T, Laufs P, Belcram K, Mizukami Y, Traas J (2004) In vivo analysis of cell division, cell growth, and differentiation at the shoot apical meristem in arabidopsis. Plant Cell **16:** 74-87

Guo MJ, Thomas J, Collins G, Timmermans MCP (2008) Direct repression of KNOX loci by the ASYMMETRIC LEAVES1 complex of Arabidopsis. Plant Cell **20:** 48-58

Han P, Li Q, Zhu YX (2008) Mutation of Arabidopsis BARD1 causes meristem defects by failing to confine WUSCHEL expression to the organizing center. Plant Cell **20:** 1482-1493

Hanano S, Domagalska MA, Nagy F, Davis SJ (2006) Multiple phytohormones influence distinct parameters of the plant circadian clock. Genes to Cells **11:** 1381-1392

Hargreaves DC, Crabtree GR (2011) ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell Research **21:** 396-420

Hay A, Kaur H, Phillips A, Hedden P, Hake S, Tsiantis M (2002) The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. Current Biology **12:** 1557-1565

Heisler MG, Ohno C, Das P, Sieber P, Reddy GV, Long JA, Meyerowitz EM (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. Current Biology **15:** 1899-1911

Helliwell CA, Wood CC, Robertson M, Peacock WJ, Dennis ES (2006) The Arabidopsis FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. Plant Journal **46:** 183-192

Hepworth SR, Valverde F, Ravenscroft D, Mouradov A, Coupland G (2002) Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. Embo Journal **21:** 4327-4337

Heyl A, Ramireddy E, Brenner WG, Riefler M, Allemeersch J, Schmulling T (2008) The transcriptional repressor ARR1-SRDX suppresses pleiotropic cytokinin activities in Arabidopsis. Plant Physiol **147:** 1380-1395

Heyl A, Wulfetange K, Pils B, Nielsen N, Romanov GA, Schmulling T (2007) Evolutionary proteomics identifies amino acids essential for ligand-binding of the cytokinin receptor CHASE domain. Bmc Evolutionary Biology **7**

Hibara K, Karim MR, Takada S, Taoka KI, Furutani M, Aida M, Tasaka M (2006) Arabidopsis CUP-SHAPED COTYLEDON3 regulates postembryonic shoot meristem and organ boundary formation. Plant Cell **18:** 2946-2957

Hibara K, Takada S, Tasaka M (2003) CUC1 gene activates the expression of SAM-related genes to induce adventitious shoot formation. Plant Journal **36:** 687-696

Higuchi M, Pischke MS, Mähönen AP, Miyawaki K, Hashimoto Y, Seki M, Kobayashi M, Shinozaki K, Kato T, Tabata S, Helariutta Y, Sussman MR, Kakimoto T (2004) *In planta* functions of the *Arabidopsis* cytokinin receptor family. Proceedings of the National Academy of Sciences of the United States of America **101:** 8821-8826

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H (2008) Regulation of cytokinin biosynthesis, compartmentalization and translocation. Journal of Experimental Botany **59:** 75-83

Holst K, Schmulling T, Werner T (2011) Enhanced cytokinin degradation in leaf primordia of transgenic Arabidopsis plants reduces leaf size and shoot organ primordia formation. Journal of Plant Physiology **168:** 1328-1334

Honma T, Goto K (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature **409:** 525-529

Horiguchi G, Kim G-T, Tsukaya H (2005) The transcription factor AtGRF5 and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of *Arabidopsis thaliana*. Plant Journal **43:** 68-78

Horiguchi G, Nakayama H, Ishikawa N, Kubo M, Demura T, Fukuda H, Tsukaya H (2011) ANGUSTIFOLIA3 Plays Roles in Adaxial/Abaxial Patterning and Growth in Leaf Morphogenesis. Plant and Cell Physiology **52:** 112-124

Hosoda K, Imamura A, Katoh E, Hatta T, Tachiki M, Yamada H, Mizuno T, Yamazaki T (2002) Molecular structure of the GARP family of plant Myb-related DNA binding motifs of the Arabidopsis response regulators. Plant Cell **14:** 2015-2029

Hu Y, Xie Q, Chua N-H (2003) The Arabidopsis auxin-inducible gene *ARGOS* controls lateral organ size. Plant Cell **15:** 1951-1961

Hurtado L, Farrona S, Reyes JC (2006) The putative SWI/SNF complex subunit BRAHMA activates flower homeotic genes in Arabidopsis thaliana. Plant Mol Biol **62:** 291-304

Hutchison CE, Li J, Argueso C, Gonzalez M, Lee E, Lewis MW, Maxwell BB, Perdue TD, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2006) The Arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. Plant Cell **18:** 3073-3087

Hwang I, Sheen J (2001) Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature **413:** 383-389

Hwang I, Sheen J, Muller B (2012) Cytokinin signaling networks. Annu Rev Plant Biol **63:** 353-380

Imaizumi T (2010) Arabidopsis circadian clock and photoperiodism: time to think about location. Current Opinion in Plant Biology **13:** 83-89

Imamura A, Hanaki N, Nakamura A, Suzuki T, Taniguchi M, Kiba T, Ueguchi C, Sugiyama T, Mizuno T (1999) Compilation and characterization of Arabidopsis thaliana response regulators implicated in His-Asp phosphorelay signal transduction. Plant and Cell Physiology **40:** 733-742

Imamura A, Kiba T, Tajima Y, Yamashino T, Mizuno T (2003) In vivo and in vitro characterization of the ARR11 response regulator implicated in the His-to-Asp phosphorelay signal transduction in Arabidopsis thaliana. Plant and Cell Physiology **44:** 122-131

Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T (2001) Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature **409:** 1060-1063

Ishida K, Yamashino T, Yokoyama A, Mizuno T (2008) Three type-B response regulators, ARR1, ARR10 and ARR12, play essential but redundant roles in cytokinin signal transduction throughout the life cycle of Arabidopsis thaliana. Plant and Cell Physiology **49:** 47-57

Jaeger KE, Wigge PA (2007) FT protein acts as a long-range signal in Arabidopsis. Current Biology **17:** 1050-1054

Jang IC, Henriques R, Seo HS, Nagatani A, Chua NH (2010) Arabidopsis PHYTOCHROME INTERACTING FACTOR Proteins Promote Phytochrome B Polyubiquitination by COP1 E3 Ligase in the Nucleus. Plant Cell **22:** 2370-2383

Jarillo JA, Pineiro M (2011) Timing is everything in plant development. The central role of floral repressors. Plant Science **181:** 364-378

Jarillo JA, Pineiro M, Cubas P, Martinez-Zapater JM (2009) Chromatin remodeling in plant development. International Journal of Developmental Biology **53:** 1581-1596

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P, Tsiantis M (2005) KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. Current Biology **15:** 1560-1565

Jerzmanowski A (2007) SWI/SNF chromatin remodeling and linker histones in plants. Biochimica Et Biophysica Acta-Gene Structure and Expression **1769:** 330-345

Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant MicroRNAs and their targets, including a stress-induced miRNA. Molecular Cell **14:** 787-799

Jonsson H, Heisler MG, Shapiro BE, Meyerowitz EM, Mjolsness E (2006) An auxin-driven polarized transport model for phyllotaxis. Proceedings of the National Academy of Sciences of the United States of America **103:** 1633-1638

Kakimoto T (2001) Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate : ATP/ADP isopentenyltransferases. Plant and Cell Physiology **42:** 677-685

Kandasamy MK, Deal RB, McKinney EC, Meagher RB (2005a) Silencing the nuclear actin-related protein AtARP4 in Arabidopsis has multiple effects on plant development, including early flowering and delayed floral senescence. Plant Journal **41:** 845-858

Kandasamy MK, McKinney EC, Deal RB, Meagher RB (2005b) Arabidopsis ARP7 is an essential actin-related protein required for normal embryogenesis, plant architecture, and floral organ abscission. Plant Physiol **138:** 2019-2032

Katz A, Oliva M, Mosquna A, Hakim O, Ohad N (2004) FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. Plant Journal **37:** 707-719

Kiba T, Yamada H, Sato S, Kato T, Tabata S, Yamashino T, Mizuno T (2003) The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in Arabidopsis thaliana. Plant and Cell Physiology **44:** 868-874

Kieffer M, Master V, Waites R, Davies B (2011) TCP14 and TCP15 affect internode length and leaf shape in Arabidopsis. Plant Journal **68:** 147-158

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **103:** 814-819

Kim JH, Choi D, Kende H (2003) The AtGRF family of putative transcription factors is involved in leaf and cotyledon growth in *Arabidopsis*. Plant Journal **36:** 94-104

Kim JH, Kende H (2004) A transcriptional coactivator, AtGIF1, is involved in regulating leaf growth and morphology in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **101:** 13374-13379

Kim JH, Lee BH (2006) GROWTH-REGULATING FACTOR4 of Arabidopsis thaliana is required for development of leaves, cotyledons, and shoot apical meristem. Journal of Plant Biology **49:** 463-468

Kinoshita T, Harada JJ, Goldberg RB, Fischer RL (2001) Polycomb repression of flowering during early plant development. Proceedings of the National Academy of Sciences of the United States of America **98:** 14156-14161

Knoll AH, Niklas KJ (1987) Adaptation, Plant Evolution, and the Fossil Record. Review of Palaeobotany and Palynology **50:** 127-149

Kohler C, Aichinger E (2010) Antagonizing Polycomb group-mediated gene repression by chromatin remodelers. Epigenetics **5:** 20-23

Kohler C, Villar CBR (2008) Programming of gene expression by Polycomb group proteins. Trends in Cell Biology **18:** 236-243

Koornneef M, Alonso-Blanco C, Blankestijn-de Vries H, Hanhart CJ, Peeters AJM (1998) Genetic interactions among late-flowering mutants of Arabidopsis. Genetics **148:** 885-892

Kouzarides T (2007) Chromatin modifications and their function. Cell **128:** 693-705

Kowalska M, Galuszka P, Frebortova J, Sebela M, Beres T, Hluska T, Smehilova M, Bilyeu KD, Frebort I (2010) Vacuolar and cytosolic cytokinin dehydrogenases of Arabidopsis thaliana Heterologous expression, purification and properties. Phytochemistry **71:** 1970-1978

Koyama T, Furutani M, Tasaka M, Ohme-Takagi M (2007) TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in Arabidopsis. Plant Cell **19:** 473-484

Koyama T, Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2010) TCP Transcription Factors Regulate the Activities of ASYMMETRIC LEAVES1 and miR164, as Well as the Auxin Response, during Differentiation of Leaves in Arabidopsis. Plant Cell **22:** 3574-3588

Krizek BA (2009) *AINTEGUMENTA* and *AINTEGUMENTA-LIKE6* act redundantly to regulate Arabidopsis floral growth and patterning. Plant Physiology **150:** 1916-1929

Krizek BA (2011) Auxin regulation of Arabidopsis flower development involves members of the AINTEGUMENTA-LIKE/PLETHORA (AIL/PLT) family. J Exp Bot **62:** 3311-3319

Kudo T, Kiba T, Sakakibara H (2010) Metabolism and Long-distance Translocation of Cytokinins. Journal of Integrative Plant Biology **52:** 53-60

Kumaran MK, Bowman JL, Sundaresan V (2002) YABBY polarity genes mediate the repression of KNOX homeobox genes in Arabidopsis. Plant Cell **14:** 2761-2770

Kurakawa T, Ueda N, Maekawa M, Kobayashi K, Kojima M, Nagato Y, Sakakibara H, Kyozuka J (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445:** 652-655

Kuroha T, Tokunaga H, Kojima M, Ueda N, Ishida T, Nagawa S, Fukuda H, Sugimoto K, Sakakibara H (2009) Functional Analyses of LONELY GUY Cytokinin-Activating Enzymes Reveal the Importance of the Direct Activation Pathway in Arabidopsis. Plant Cell **21:** 3152-3169

Kwiatkowska D (2006) Flower primordium formation at the Arabidopsis shoot apex: quantitative analysis of surface geometry and growth. J Exp Bot **57:** 571-580

Kwon CS, Chen C, Wagner D (2005) *WUSCHEL* is a primary target for transcriptional regulation by SPLAYED in dynamic control of stem cell fate in *Arabidopsis*. Genes & Development **19:** 992-1003

Kwon CS, Hibara K, Pfluger J, Bezhani S, Metha H, Aida M, Tasaka M, Wagner D (2006) A role for chromatin remodeling in regulation of CUC gene expression in the Arabidopsis cotyledon boundary. Development **133:** 3223-3230

Kwon CS, Wagner D (2007) Unwinding chromatin for development and growth: a few genes at a time. Trends in Genetics **23:** 403-412

Lau OS, Deng XW (2010) Plant hormone signaling lightens up: integrators of light and hormones. Current Opinion in Plant Biology **13:** 571-577

Laufs P, Peaucelle A, Morin H, Traas J (2004) MicroRNA regulation of the CUC genes is required for boundary size control in Arabidopsis meristems. Development **131:** 4311-4322

Leakey ADB, Lau JA (2012) Evolutionary context for understanding and manipulating plant responses to past, present and future atmospheric [CO2]. Philosophical Transactions of the Royal Society B-Biological Sciences **367:** 613-629

Lee BH, Ko J-H, Lee S, Lee Y, Pak J-H, Kim JH (2009) The Arabidopsis *GRF-INTERACTING FACTOR* gene family performs an overlapping function in determining organ size as well as multiple developmental properties. Plant Physiology **151:** 655-668

Lee DJ, Kim S, Ha YM, Kim J (2008a) Phosphorylation of Arabidopsis response regulator 7 (ARR7) at the putative phospho-accepting site is required for ARR7 to act as a negative regulator of cytokinin signaling. Planta **227:** 577-587

Lee DJ, Park JY, Ku SJ, Ha YM, Kim S, Kim MD, Oh MH, Kim J (2007) Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7(ARR7) overexpression in cytokinin response. Molecular Genetics and Genomics **277:** 115-137

Lee J, Oh M, Park H, Lee I (2008b) SOC1 translocated to the nucleus by interaction with AGL24 directly regulates LEAFY. Plant Journal **55:** 832-843

Leibfried A, To JPC, Busch W, Stehling S, Kehle A, Demar M, Kieber JJ, Lohmann JU (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438:** 1172-1175

Leivar P, Monte E, Oka Y, Liu T, Carle C, Castillon A, Huq E, Quail PH (2008) Multiple phytochrome-interacting bHLH transcription factors repress premature seedling photomorphogenesis in darkness. Current Biology **18:** 1815-1823

Leivar P, Quail PH (2011) PIFs: pivotal components in a cellular signaling hub. Trends in Plant Science **16:** 19-28

Leivar P, Tepperman JM, Cohn MM, Monte E, Al-Sady B, Erickson E, Quail PH (2012) Dynamic Antagonism between Phytochromes and PIF Family Basic Helix-Loop-Helix Factors Induces Selective Reciprocal Responses to Light and Shade in a Rapidly Responsive Transcriptional Network in Arabidopsis. Plant Cell **24:** 1398-1419

Li CX, Potuschak T, Colon-Carmona A, Gutierrez RA, Doerner P (2005) Arabidopsis TCP20 links regulation of growth and cell division control pathways. Proceedings of the National Academy of Sciences of the United States of America **102:** 12978-12983

Li Y, Zheng L, Corke F, Smith C, Bevan MW (2008) Control of final seed and organ size by the *DA1* gene family in *Arabidopsis thaliana*. Genes & Development **22:** 1331-1336

Li Z, Li B, Shen WH, Huang H, Dong A (2012a) TCP transcription factors interact with AS2 in the repression of class-I KNOX genes in Arabidopsis thaliana. Plant J **71:** 99-107

Li ZY, Li B, Dong AW (2012b) The Arabidopsis Transcription Factor AtTCP15 Regulates Endoreduplication by Modulating Expression of Key Cell-cycle Genes. Mol Plant **5:** 270-280

Lincoln C, Long J, Yamaguchi J, Serikawa K, Hake S (1994) A Knotted1-Like Homeobox Gene in Arabidopsis Is Expressed in the Vegetative Meristem and Dramatically Alters Leaf Morphology When Overexpressed in Transgenic Plants. Plant Cell **6:** 1859-1876

Lindsay DL, Sawhney VK, Bonham-Smith PC (2006) Cytokinin-induced changes in CLAVATA1 and WUSCHEL expression temporally coincide with altered floral development in Arabidopsis. Plant Science **170:** 1111-1117

Liu D, Song Y, Chen Z, Yu D (2009) Ectopic expression of miR396 suppresses *GRF* target gene expression and alters leaf growth in *Arabidopsis*. Physiologia Plantarum **136:** 223-236

Lohrmann J, Buchholz G, Keitel C, Sweere U, Kircher S, Baurle I, Kudla J, Schafer E, Harter K (1999) Differential expression and nuclear localization of response regulator-like proteins from Arabidopsis thaliana. Plant Biology **1:** 495-505

Long J, Barton MK (2000) Initiation of axillary and floral meristems in Arabidopsis. Developmental Biology **218:** 341-353

Long JA, Moan EI, Medford JI, Barton MK (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature **379:** 66-69

Mahonen AP, Bonke M, Kauppinen L, Riikonen M, Benfey PN, Helariutta Y (2000) A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. Genes & Development **14:** 2938-2943

Makarevich G, Leroy O, Akinci U, Schubert D, Clarenz O, Goodrich J, Grossniklaus U, Kohler C (2006) Different Polycomb group complexes regulate common target genes in Arabidopsis. Embo Reports **7:** 947-952

Martin-Trillo M, Cubas P (2010) TCP genes: a family snapshot ten years later. Trends in Plant Science **15:** 31-39

Mason MG, Mathews DE, Argyros DA, Maxwell BB, Kieber JJ, Alonso JM, Ecker JR, Schaller GE (2005) Multiple type-B response regulators mediate cytokinin signal transduction in *Arabidopsis*. Plant Cell **17:** 3007-3018

Maston GA, Evans SK, Green MR (2006) Transcriptional regulatory elements in the human genome. Annual Review of Genomics and Human Genetics **7:** 29-59

Mathieu J, Warthmann N, Kuttner F, Schmid M (2007) Export of FT protein from phloem companion cells is sufficient for floral induction in Arabidopsis. Current Biology **17:** 1055-1060

Matsumoto-Kitano M, Kusumoto T, Tarkowski P, Kinoshita-Tsujimura K, Vaclavikova K, Miyawaki K, Kakimoto T (2008) Cytokinins are central regulators of cambial activity. Proceedings of the National Academy of Sciences of the United States of America **105:** 20027-20031

Mayer KFX, Schoof H, Haecker A, Lenhard M, Jürgens G, Laux T (1998) Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell **95:** 805-815

Meagher RB, Deal RB, Kandasamy MK, McKinney EC (2005) Nuclear actin-related proteins as epigenetic regulators of development. Plant Physiol **139:** 1576-1585

Miller CO, Skoog F, Okumura FS, Vonsaltza MH, Strong FM (1955a) Structure and Synthesis of Kinetin. Journal of the American Chemical Society **77:** 2662-2663

Miller CO, Skoog F, Vonsaltza MH, Strong FM (1955b) Kinetin, a Cell Division Factor from Deoxyribonucleic Acid. Journal of the American Chemical Society **77:** 1392-1392

Mira-Rodado V, Sweere U, Grefen C, Kunkel T, Fejes E, Nagy F, Schafer E, Harter K (2007) Functional cross-talk between two-component and phytochrome B signal transduction in Arabidopsis. J Exp Bot **58:** 2595-2607

Miyata S, Urao T, Yamaguchi-Shinozaki K, Shinozaki K (1998) Characterization of genes for two-component phosphorelay mediators with a single HPt domain in Arabidopsis thaliana. Febs Letters **437:** 11-14

Miyawaki K, Matsumoto-Kitano M, Kakimoto T (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant Journal **37:** 128-138

Miyawaki K, Tarkowski P, Matsumoto-Kitano M, Kato T, Sato S, Tarkowska D, Tabata S, Sandberg G, Kakimoto T (2006) Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proceedings of the National Academy of Sciences of the United States of America **103:** 16598-16603

Mizukami Y, Fischer RL (2000) Plant organ size control: *AINTEGUMENTA* regulates growth and cell numbers during organogenesis. Proceedings of the National Academy of Sciences of the United States of America **97:** 942-947

Mok DW, Mok MC (2001) Cytokinin Metabolism and Action. Annu Rev Plant Physiol Plant Mol Biol **52:** 89-118

Mok M (1994) Cytokinins and plant development: an overview. *In* D Mok, M Mok, eds, Cytokinins: chemistry, activity, and function, Boca Raton, FL: CRC, pp 155-166

Moon J, Hake S (2011) How a leaf gets its shape. Current Opinion in Plant Biology **14:** 24-30

Moon J, Suh SS, Lee H, Choi KR, Hong CB, Paek NC, Kim SG, Lee I (2003) The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. Plant Journal **35:** 613-623

Mudunkothge JS, Krizek BA (2012) Three Arabidopsis AIL/PLT genes act in combination to regulate shoot apical meristem function. Plant J **71:** 108-121

Müller B, Sheen J (2008) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature **453:** 1094-1097

Nakahigashi K, Jasencakova Z, Schubert I, Goto K (2005) The Arabidopsis HETEROCHROMATIN PROTEIN1 homolog (TERMINAL FLOWER2) silences genes within the euchromatic region but not genes positioned in heterochromatin. Plant and Cell Physiology **46:** 1747-1756

Neigeborn L, Carlson M (1984) Genes Affecting the Regulation of Suc2 Gene-Expression by Glucose Repression in Saccharomyces-Cerevisiae. Genetics **108:** 845-858

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. Plant Cell **16:** 1365-1377

Nole-Wilson S, Krizek BA (2006) AINTEGUMENTA contributes to organ polarity and regulates growth of lateral organs in combination with YABBY genes. Plant Physiol **141:** 977-987

Nole-Wilson S, Tranby TL, Krizek BA (2005) *AINTEGUMENTA*-like (*AIL*) genes are expressed in young tissues and may specify meristematic or division-competent states. Plant Molecular Biology **57:** 613-628

Ogawa M, Shinohara H, Sakagami Y, Matsubayashi Y (2008) *Arabidopsis* CLV3 peptide directly binds CLV1 ectodomain. Science **319:** 294

Oh E, Kang H, Yamaguchi S, Park J, Lee D, Kamiya Y, Choi G (2009) Genome-wide analysis of genes targeted by PHYTOCHROME INTERACTING FACTOR 3-LIKE5 during seed germination in *Arabidopsis*. Plant Cell **21:** 403-419

Oh E, Yamaguchi S, Hu J, Yusuke J, Jung B, Paik I, Lee H-S, Sun T-p, Kamiya Y, Choi G (2007) PIL5, a phytochrome-interacting bHLH protein, regulates gibberellin responsiveness by binding directly to the *GAI* and *RGA* promoters in *Arabidopsis* seeds. Plant Cell **19:** 1192-1208

Oh E, Yamaguchi S, Kamiya Y, Bae G, Chung W-I, Choi G (2006) Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in Arabidopsis. Plant Journal **47:** 124-139

Ori N, Eshed Y, Chuck G, Bowman JL, Hake S (2000) Mechanisms that control knox gene expression in the Arabidopsis shoot. Development **127:** 5523-5532

Osborne CP, Beerling DJ, Lomax BH, Chaloner WG (2004) Biophysical constraints on the origin of leaves inferred from the fossil record. Proc Natl Acad Sci U S A **101:** 10360-10362

Osterlund MT, Hardtke CS, Wei N, Deng XW (2000) Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*. Nature **405:** 462-466

Oyama T, Shimura Y, Okada K (1997) The *Arabidopsis HY5* gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes & Development **11:** 2983-2995

Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D (2003) Control of leaf morphogenesis by microRNAs. Nature **425:** 257-263

Pautot W, Dockx J, Hamant O, Kronenberger J, Grandjean O, Jublot D, Traas J (2001) KNAT2: Evidence for a link between knotted-like genes and carpel development. Plant Cell **13:** 1719-1734

Pelaz S, Tapia-Lopez R, Alvarez-Buylla ER, Yanofsky MF (2001) Conversion of leaves into petals in Arabidopsis. Current Biology **11:** 182-184

Perales M, Reddy GV (2012) Stem cell maintenance in shoot apical meristems. Current Opinion in Plant Biology **15:** 10-16

Pien S, Grossniklaus U (2007) Polycomb group and trithorax group proteins in Arabidopsis. Biochimica Et Biophysica Acta-Gene Structure and Expression **1769:** 375-382

Prasad K, Grigg SP, Barkoulas M, Yadav RK, Sanchez-Perez GF, Pinon V, Blilou I, Hofhuis H, Dhonukshe P, Galinha C, Mahonen AP, Muller WH, Raman S, Verkleij AJ, Snel B, Reddy GV, Tsiantis M, Scheres B (2011) Arabidopsis PLETHORA Transcription Factors Control Phyllotaxis. Current Biology **21:** 1123-1128

Rashotte AM, Goertzen LR (2010) The CRF domain defines Cytokinin Response Factor proteins in plants. Bmc Plant Biology **10**

Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2006) A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proceedings of the National Academy of Sciences of the United States of America **103:** 11081-11085

Raven JA, Edwards D (2001) Roots: evolutionary origins and biogeochemical significance. J Exp Bot **52:** 381-401

Reinhardt D, Pesce E-R, Stieger P, Mandel T, Baltensperger K, Bennett M, Traas J, Friml J, Kuhlemeier C (2003) Regulation of phyllotaxis by polar auxin transport. Nature **426:** 255-260

Riefler M, Novak O, Strnad M, Schmulling T (2006) Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18:** 40-54

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH (1999) Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science **283:** 1541-1544

Rizzini L, Favory JJ, Cloix C, Faggionato D, O'Hara A, Kaiserli E, Baumeister R, Schafer E, Nagy F, Jenkins GI, Ulm R (2011) Perception of UV-B by the Arabidopsis UVR8 Protein. Science **332:** 103-106

Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396. Development **137:** 103-112

Roudier F, Teixeira FK, Colot V (2009) Chromatin indexing in Arabidopsis: an epigenomic tale of tails and more. Trends in Genetics **25:** 511-517

Royer DL (2006) CO2-forced climate thresholds during the Phanerozoic. Geochimica Et Cosmochimica Acta **70:** 5665-5675

Royer DL, Berner RA, Beerling DJ (2001) Phanerozoic atmospheric CO2 change: evaluating geochemical and paleobiological approaches. Earth-Science Reviews **54:** 349-392

Rupp HM, Frank M, Werner T, Strnad M, Schmulling T (1999) Increased steady state mRNA levels of the STM and KNAT1 homeobox genes in cytokinin overproducing Arabidopsis thaliana indicate a role for cytokinins in the shoot apical meristem. Plant Journal **18:** 557-563

Sakai H, Aoyama T, Bono H, Oka A (1998) Two-component response regulators from Arabidopsis thaliana contain a putative DNA-binding motif. Plant and Cell Physiology **39:** 1232-1239

Sakai H, Aoyama T, Oka A (2000) Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. Plant Journal **24:** 703-711

Sakai H, Honma T, Aoyama T, Sato S, Kato T, Tabata S, Oka A (2001) ARR1, a transcription factor for genes immediately responsive to cytokinins. Science **294:** 1519-1521

Saleh A, Al-Abdallat A, Ndamukong I, Alvarez-Venegas R, Avramova Z (2007) The Arabidopsis homologs of trithorax (ATX1) and enhancer of zeste (CLF) establish 'bivalent chromatin marks' at the silent AGAMOUS locus. Nucleic Acids Research **35:** 6290-6296

Salome PA, To JPC, Kieber JJ, McClung CR (2006) Arabidopsis response regulators ARR3 and ARR4 play cytokinin-independent roles in the control of circadian period. Plant Cell **18:** 55-69

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science **288:** 1613-1616

Sampedro J, Cosgrove DJ (2005) The expansin superfamily. Genome Biology **6:** 242

Sarnowski TJ, Rios G, Jasik J, Swiezewski S, Kaczanowski S, Li Y, Kwiatkowska A, Pawlikowska K, Kozbial M, Kozbial P, Koncz C, Jerzmanowski A (2005) SWI3 subunits of putative SWI/SNF

chromatin-remodeling complexes play distinct roles during Arabidopsis development. Plant Cell **17:** 2454-2472

Sarnowski TJ, Swiezewski S, Pawlikowska K, Kaczanowski S, Jerzmanowski A (2002) AtSWI3B, an Arabidopsis homolog of SWI3, a core subunit of yeast Swi/Snf chromatin remodeling complex, interacts with FCA, a regulator of flowering time. Nucleic Acids Research **30:** 3412-3421

Sarojam R, Sappl PG, Goldshmidt A, Efroni I, Floyd SK, Eshed Y, Bowman JL (2010) Differentiating Arabidopsis Shoots from Leaves by Combined YABBY Activities. Plant Cell **22:** 2113-2130

Schommer C, Palatnik JF, Aggarwal P, Chételat A, Cubas P, Farmer EE, Nath U, Weigel D (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. PLoS Biology **6:** e230

Schoof H, Lenhard M, Haecker A, Mayer KFX, Jürgens G, Laux T (2000) The stem cell population of *Arabidopsis* shoot meristems in maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. Cell **100:** 635-644

Schruff MC, Spielman M, Tiwari S, Adams S, Fenby N, Scott RJ (2006) The *AUXIN RESPONSE FACTOR 2* gene of *Arabidopsis* links auxin signalling, cell division, and the size of seeds and other organs. Development **133:** 251-261

Schubert D, Primavesi L, Bishopp A, Roberts G, Doonan J, Jenuwein T, Goodrich J (2006) Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. Embo Journal **25:** 4638-4649

Searle I, He YH, Turck F, Vincent C, Fornara F, Krober S, Amasino RA, Coupland G (2006) The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. Genes & Development **20:** 898-912

Semiarti E, Ueno Y, Tsukaya H, Iwakawa H, Machida C, Machida Y (2001) The asymmetric leaves2 gene of Arabidopsis thaliana regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. Development **128:** 1771-1783

Shen Y, Khanna R, Carle CM, Quail PH (2007) Phytochrome induces rapid PIF5 phosphorylation and degradation in response to red-light activation. Plant Physiology **145:** 1043-1051

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symposia of the Society for Experimental Biology **11:** 118-130

Srikanth A, Schmid M (2011) Regulation of flowering time: all roads lead to Rome. Cellular and Molecular Life Sciences **68:** 2013-2037

Steiner E, Efroni I, Gopalraj M, Saathoff K, Tseng TS, Kieffer M, Eshed Y, Olszewski N, Weiss D (2012) The Arabidopsis O-Linked N-Acetylglucosamine Transferase SPINDLY Interacts with Class I TCPs to Facilitate Cytokinin Responses in Leaves and Flowers. Plant Cell **24:** 96-108

Stern M, Jensen R, Herskowitz I (1984) 5 Swi Genes Are Required for Expression of the Ho Gene in Yeast. Journal of Molecular Biology **178:** 853-868

Stolz A, Riefler M, Lomin SN, Achazi K, Romanov GA, Schmulling T (2011) The specificity of cytokinin signalling in Arabidopsis thaliana is mediated by differing ligand affinities and expression profiles of the receptors. Plant Journal **67:** 157-168

Su Y, Kwon CS, Bezhani S, Huvermann B, Chen C, Peragine A, Kennedy JF, Wagner D (2006) The N-terminal ATPase AT-hook-containing region of the Arabidopsis chromatin-remodeling protein SPLAYED is sufficient for biological activity. Plant J **46:** 685-699

Suarez-Lopez P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G (2001) CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature **410:** 1116-1120

Suzuki T, Imamura A, Ueguchi C, Mizuno T (1998) Histidine-containing phosphotransfer (HPt) signal transducers implicated in His-to-Asp phosphorelay in Arabidopsis. Plant and Cell Physiology **39:** 1258-1268

Suzuki T, Miwa K, Ishikawa K, Yamada H, Aiba H, Mizuno T (2001a) The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. Plant and Cell Physiology **42:** 107-113

Suzuki T, Sakurai K, Imamura A, Nakamura A, Ueguchi C, Mizuno T (2000) Compilation and characterization of histidine-containing phosphotransmitters implicated in His-to-Asp phosphorelay in plants: AHP signal transducers of Arabidopsis thaliana. Bioscience Biotechnology and Biochemistry **64:** 2486-2489

Suzuki T, Sakurai K, Ueguchi C, Mizuno T (2001b) Two types of putative nuclear factors that physically interact with histidine-containing phosphotransfer (Hpt) domains, signaling mediators in His-to-Asp phosphorelay, in Arabidopsis thaliana. Plant and Cell Physiology **42:** 37-45

Sweere U, Eichenberg K, Lohrmann J, Mira-Rodado V, Baurle I, Kudla J, Nagy F, Schafer E, Harter K (2001) Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. Science **294:** 1108-1111

Takada S, Hibara K, Ishida T, Tasaka M (2001) The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. Development **128:** 1127-1135

Takei K, Sakakibara H, Sugiyama T (2001a) Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in Arabidopsis thaliana. Journal of Biological Chemistry **276:** 26405-26410

Takei K, Sakakibara H, Taniguchi M, Sugiyama T (2001b) Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: Implication of cytokinin species that induces gene expression of maize response regulator. Plant and Cell Physiology **42:** 85-93

Takei K, Yamaya T, Sakakibara H (2004) Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-Zeatin. Journal of Biological Chemistry **279:** 41866-41872

Tang XR, Hou AF, Babu M, Nguyen V, Hurtado L, Lu Q, Reyes JC, Wang AM, Keller WA, Harada JJ, Tsang EWT, Cui YH (2008) The Arabidopsis BRAHMA chromatin-remodeling ATPase is involved in repression of seed maturation genes in leaves. Plant Physiol **147:** 1143-1157

Taniguchi M, Sasaki N, Tsuge T, Aoyama T, Oka A (2007) ARR1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions. Plant and Cell Physiology **48:** 263-277

To JPC, Deruère J, Maxwell BB, Morris VF, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2007) Cytokinin regulates type-A *Arabidopsis* Response Regulator activity and protein stability via two-component phosphorelay. Plant Cell **19:** 3901-3914

To JPC, Haberer G, Ferreira FJ, Deruere J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2004) Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. Plant Cell **16:** 658-671

Tokunaga H, Kojima M, Kuroha T, Ishida T, Sugimoto K, Kiba T, Sakakibara H (2012) Arabidopsis lonely guy (LOG) multiple mutants reveal a central role of the LOG-dependent pathway in cytokinin activation. Plant Journal **69:** 355-365

Tsukaya H (2008) Controlling size in multicellular organs: focus on the leaf. PLoS Biology **6:** e174

Turck F, Roudier F, Farrona S, Martin-Magniette ML, Guillaume E, Buisine N, Gagnot S, Martienssen RA, Coupland G, Colot V (2007) Arabidopsis TFL2/LHP1 specifically associates with genes marked by trimethylation of histone H3 lysine 27. Plos Genetics **3:** 855-866

Ueguchi C, Koizumi H, Suzuki T, Mizuno T (2001) Novel family of sensor histidine kinase genes in Arabidopsis thaliana. Plant and Cell Physiology **42:** 231-235

Vaillant I, Paszkowski J (2007) Role of histone and DNA methylation in gene regulation. Current Opinion in Plant Biology **10:** 528-533

Vandenbussche F, Habricot Y, Condiff AS, Maldiney R, Van der Straeten D, Ahmad M (2007) HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in *Arabidopsis thaliana*. Plant Journal **49:** 428-441

Vidaurre DP, Ploense S, Krogan NT, Berleth T (2007) AMP1 and MP antagonistically regulate embryo and meristem development in Arabidopsis. Development **134:** 2561-2567

Vroemen CW, Mordhorst AP, Albrecht C, Kwaaitaal MACJ, de Vries SC (2003) The CUP-SHAPED COTYLEDON3 gene is required for boundary and shoot meristem formation in Arabidopsis. Plant Cell **15:** 1563-1577

Wagner D (2003) Chromatin regulation of plant development. Current Opinion in Plant Biology **6:** 20-28

Wagner D, Meyerowitz EM (2002) SPLAYED, a novel SWI/SNF ATPase homolog, controls reproductive development in Arabidopsis. Current Biology **12:** 85-94

Wang H, Deng XW (2004) Phytochrome signaling mechanism. Arabidopsis Book **3**

Wang L, Gu X, Xu D, Wang W, Wang H, Zeng M, Chang Z, Huang H, Cui X (2011) miR396-targeted AtGRF transcription factors are required for coordination of cell division and differentiation during leaf development in Arabidopsis. J Exp Bot **62:** 761-773

Wareing PF, Khalifa MM, Treharne KJ (1968) Rate-Limiting Processes in Photosynthesis at Saturating Light Intensities. Nature **220:** 453-&

Went FW (1928) Wuchsstoff und wachstum. Rec Trav Bot Neerl **25:** 1-116

Werner T, Holst K, Pors Y, Guivarc'h A, Mustroph A, Chriqui D, Grimm B, Schmulling T (2008) Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. J Exp Bot **59:** 2659-2672

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmulling T (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15:** 2532-2550

White DWR (2006) *PEAPOD* regulates lamina size and curvature in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **103:** 13238-13243

Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. Science **309:** 1056-1059

Wilson BG, Roberts CWM (2011) SWI/SNF nucleosome remodellers and cancer. Nature Reviews Cancer **11:** 481-492

Winter CM, Austin RS, Blanvillain-Baufume S, Reback MA, Monniaux M, Wu MF, Sang Y, Yamaguchi A, Yamaguchi N, Parker JE, Parcy F, Jensen ST, Li HZ, Wagner D (2011) LEAFY Target Genes Reveal Floral Regulatory Logic, cis Motifs, and a Link to Biotic Stimulus Response. Developmental Cell **20:** 430-443

Wiśniewska J, Xu J, Seifertová D, Brewer PB, Růžička K, Blilou I, Rouquié D, Benková E, Scheres B, Friml J (2006) Polar PIN localization directs auxin flow in plants. Science **312:** 883

Wolters H, Jurgens G (2009) Survival of the flexible: hormonal growth control and adaptation in plant development. Nature Reviews Genetics **10:** 305-317

Wu MF, Sang Y, Bezhani S, Yamaguchi N, Han SK, Li ZT, Su YH, Slewinski TL, Wagner D (2012) SWI2/SNF2 chromatin remodeling ATPases overcome polycomb repression and control floral organ identity with the LEAFY and SEPALLATA3 transcription factors. Proceedings of the National Academy of Sciences of the United States of America **109:** 3576-3581

Wulfetange K, Lomin SN, Romanov GA, Stolz A, Heyl A, Schmulling T (2011) The Cytokinin Receptors of Arabidopsis Are Located Mainly to the Endoplasmic Reticulum. Plant Physiol **156:** 1808-1818

Xu L, Shen WH (2008) Polycomb Silencing of KNOX Genes Confines Shoot Stem Cell Niches in Arabidopsis. Current Biology **18:** 1966-1971

Yadav RK, Girke T, Pasala S, Xie MT, Reddy V (2009) Gene expression map of the Arabidopsis shoot apical meristem stem cell niche. Proceedings of the National Academy of Sciences of the United States of America **106:** 4941-4946

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant and Cell Physiology **42:** 1017-1023

Yamaguchi A, Kobayashi Y, Goto K, Abe M, Araki T (2005) TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. Plant and Cell Physiology **46:** 1175-1189

Yanai O, Shani E, Dolezal K, Tarkowski P, Sablowski R, Sandberg G, Samach A, Ori N (2005) *Arabidopsis* KNOXI proteins activate cytokinin biosynthesis. Current Biology **15:** 1566-1571

Yoon HS, Hackett JD, Ciniglia C, Pinto G, Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Mol Biol Evol **21:** 809-818

Yoshida S, Mandel T, Kuhlemeier C (2011) Stem cell activation by light guides plant organogenesis. Genes & Development **25:** 1439-1450

Yu X, Liu H, Klejnot J, Lin C (2010) The Cryptochrome Blue Light Receptors. Arabidopsis Book **8:** e0135

Zhang H, Bishop B, Ringenberg W, Muir WM, Ogas J (2012) The CHD3 Remodeler PICKLE Associates with Genes Enriched for Trimethylation of Histone H3 Lysine 27. Plant Physiol **159:** 418-432

Zhang X, Germann S, Blus BJ, Khorasanizadeh S, Gaudin V, Jacobsen SE (2007) The Arabidopsis LHP1 protein colocalizes with histone H3 Lys27 trimethylation. Nature Structural & Molecular Biology **14:** 869-871

Zhao Z, Andersen SU, Ljung K, Dolezal K, Miotk A, Schultheiss SJ, Lohmann JU (2010) Hormonal control of the shoot stem-cell niche. Nature **465:** 1089-1092

Zheng BL, Chen XM (2011) Dynamics of histone H3 lysine 27 trimethylation in plant development. Current Opinion in Plant Biology **14:** 123-129

Zheng BL, Deng Y, Mu JY, Ji ZD, Xiang TT, Niu QW, Chua NH, Zuo JR (2006) Cytokinin affects circadian-clock oscillation in a phytochrome B- and Arabidopsis response regulator 4-dependent manner. Physiologia Plantarum **127:** 277-292

Zhou CH, Miki B, Wu KQ (2003) CHB2, a member of the SWI3 gene family, is a global regulator in Arabidopsis. Plant Mol Biol **52:** 1125-1134

Zhu XG, Long SP, Ort DR (2010) Improving Photosynthetic Efficiency for Greater Yield. Annual Review of Plant Biology, Vol 61 **61:** 235-261

2

Image: Arabidopsis Col-0 flower.

Chapter 2: Combining enhanced root and shoot growth reveals crosstalk between pathways that control plant organ size in Arabidopsis

Liesbeth Vercruyssen[1,2], Nathalie Gonzalez[1,2], Tomáš Werner[3], Thomas Schmülling[3], and Dirk Inzé[1,2]

[1]Department of Plant Systems Biology, VIB, 9052 Ghent, Belgium

[2]Department of Plant Biotechnology and Genetics, Ghent University, 9052 Ghent, Belgium

[3]Institute of Biology/Applied Genetics, Dahlem Centre of Plant Sciences (DCPS), Freie Universität Berlin, 14195 Berlin, Germany

L.V. wrote the manuscript and performed all experiments. N.G., T.S. and D.I. contributed valuable comments and fruitful discussions. T.W. provided the *PYK10-CKX3* construct and assisted with text corrections.

Published in Plant Physiology, **155:** 1339-1352.

ABSTRACT

Functionally distinct Arabidopsis (*Arabidopsis thaliana*) genes that positively affect root or shoot growth when ectopically expressed, were combined to explore the feasibility of enhanced biomass production. Enhanced root growth resulting from cytokinin deficiency was obtained by overexpressing *CYTOKININ OXIDASE/DEHYDROGENASE 3* (*CKX3*) under the control of the root-specific *PYK10* promoter. Plants harboring the *PYK10-CKX3* construct were crossed with four different transgenic lines showing enhanced leaf growth. For all combinations, the phenotypic traits of the individual lines could be combined, resulting in an overall growth increase. Unexpectedly, three out of four combinations had more than additive effects. Both leaf and root growth were synergistically enhanced in plants overexpressing *CKX3* and *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*), indicating crosstalk between cytokinins and brassinosteroids. In agreement, treatment of *PYK10-CKX3* plants with brassinolide resulted in a dramatic increase in lateral root growth that could not be observed in wild-type plants. Co-expression of *CKX3* and the *GROWTH-REGULATING FACTOR 5* (*GRF5*) antagonized the effects of *GRF5* overexpression, revealing an interplay between cytokinins and *GRF5* during leaf cell proliferation. The

combined overexpression of *CKX3* and the *GIBBERELLIN 20-OXIDASE 1* (*GA20ox1*) led to a synergistic increase in leaf growth, suggesting an antagonistic growth control by cytokinins and gibberellins. Only additive effects on root and shoot growth were visible in plants ectopically expressing both *CKX3* and *ARABIDOPSIS VACUOLAR PYROPHOSPHATASE 1* (*AVP1*), hinting at an independent action mode. Our results reveal new interactions and contribute to the molecular and physiological understanding of biomass production at the whole plant level.

INTRODUCTION

As the world population is estimated to grow to 9.2 billion people by 2050, the availability of plant-derived products has to increase drastically to meet the needs not only for food, feed, and fiber but also for bio-energy and other industrial applications (Borlaug, 2007). In the future, less arable land will be available, while more crops need to be produced. To lower the negative impact on the environment, increasing plant biomass production on existing agricultural land will diminish the demand for new crop acreage (Edgerton, 2009). Whereas traditional breeding combined with improved agronomical practices will however not keep up with the increasing global demands, biotechnology can help serving this purpose (Borlaug, 2007). Marker-assisted breeding and introduction of transgenic traits for biotic and abiotic stress resistance have proven to increase crop gain (Eathington et al., 2007; Edgerton, 2009). Furthermore, improving yield by modulating endogenous molecular pathways will be an important feature of the next generation biotech crops.

The introduction of multiple transgenes has become widely adopted (Naqvi et al., 2010), e.g. three carotenoid biosynthesis genes enable provitamin A production in rice (*Oryza sativa*) cv Golden Rice (Ye et al., 2000), while as much as eight genes confer insecticide and pesticide resistance to the new biotech maize (*Zea mays*; James, 2009). The latter case demonstrates the great potential of stacking distinct phenotypic traits, which prompted us to investigate whether enhanced root growth can be combined with enhanced shoot growth to further improve plant productivity.

Plant growth is highly regulated throughout development and two main levels of coordination can be distinguished. First, the final plant size is determined by a global

growth coordination of distinct organs. This implicates that for optimal growth, energy production in photosynthetic leaves has to be fine-tuned to the availability of water and nutrients supplied by the root, inevitably linking shoot and root growth (Paul and Foyer, 2001). Since photosynthesis and source tissue development are regulated by sink tissue through the carbon nitrogen balance (Paul and Foyer, 2001), altering the source/sink balance by changing the root/shoot ratio will probably impact on the total plant growth. An enlarged root system for instance could act as a strong sink and prevent enhanced growth of the shoot.

Second, different organs are delineated by specific tissue patterns that are generated by coordinated proliferation and expansion of individual cells. The proliferation process is characterized by extensive cell division and cell size homeostasis. During the expansion phase, cells become much larger than meristematic cells and start to differentiate (Beemster et al., 2005). Roots exhibit an indeterminate growth pattern. Meristematic cells in the root tip continuously produce new cells, while older cells are subsequently displaced away from the root tip. As a result, the proliferation and expansion phases coexist in roots throughout the life cycle of the plant. In contrast, the shoot consists of lateral organs of determinate size. In the leaf of Arabidopsis, cell proliferation gradually decreases from the tip toward the base, while cell expansion progressively increases in the opposite direction (Donnelly et al., 1999). The rate and duration of both cell proliferation and expansion ultimately determine the size at the organ level.

Despite the elaborate knowledge on the physiological aspect of biomass production, the molecular understanding is limited. Over the past decades, many genes have been identified that improve organ size when overexpressed or mutated (Gonzalez et al., 2009; Krizek, 2009) and were previously referred to as 'Intrinsic Yield Genes' (IYGs). These genes belong to a diversity of functional classes and operate in different pathways, demonstrating that plant growth and yield are complex traits. However, all pathways influencing growth should eventually converge to control cell division and cell expansion, suggesting an extensive crosstalk and creating the opportunity to further improve growth by combining transgenes or better performing gene alleles that function in these pathways.

As plant hormones are involved in every aspect of plant biology, it is not surprising that a number of IYGs are part of the hormone biosynthetic or signaling pathways or exert their

effect by influencing the hormone metabolism. Cytokinin oxidase/dehydrogenase (CKX) enzymes catalyze the degradation of a number of biologically active cytokinins and, hence, contribute to the regulation of cytokinin levels (Mok and Mok, 2001; Werner et al., 2006). The Arabidopsis *CKX* family contains seven members (Schmülling et al., 2003) of which the phenotype resulting from overexpression of six (*CKX1* to *CKX6*) members has been described (Werner et al., 2003): *CKX*-overproducing plants have a reduced cytokinin content, leading to the development of the typical cytokinin deficiency syndrome characterized by an increased root growth, but severely diminished shoot growth (Werner et al., 2003).

Detailed analysis of *35S-CKX1*-overexpressing plants at the cellular level has revealed that cytokinins function in the process of cell proliferation in meristems (Werner et al., 2003). The shoot apical meristem of *CKX*-overexpressing plants is reduced in size and cell production in the leaves is diminished, which is slightly compensated for by an enlarged cell size. In contrast, the primary root apical meristem is enlarged, additional cell files are formed, and all cell types show an increased radial expansion. These observations suggest that cytokinins have a major role in regulating meristem activity by controlling the exit of cells from the meristem and hence the duration of cell proliferation (Werner et al., 2003). Moreover, in roots, cytokinins most probably act specifically at the transition zone between dividing and expanding cells to promote cell differentiation (Dello Ioio et al., 2007).

Besides a plausible local function in the same tissue where they are synthesized, cytokinins, as other plant hormones, function in long distance signaling between root and shoot tissues (Kudo et al., 2010). Evidence supports a role in nutrient signaling to coordinate metabolic processes between sink and source tissues (Argueso et al., 2009). Moreover, cytokinins stimulate photosynthesis (Wareing et al., 1968) and are able to establish local metabolic sinks or enhance sink strength (Kuiper, 1993; Guivarc'h et al., 2002). The effect on sink strength has also been demonstrated in *35S-CKX1*-overexpressing tobacco (*Nicotiana tabacum*) plants, in which decreased cytokinin levels reduced the sink strength of the shoot (Werner et al., 2008). Clearly, cytokinins play a major role in fine-tuning the distribution of assimilates in the whole plant and, hence contribute to the regulation of growth and biomass production.

Here, enhanced root growth resulting from cytokinin deficiency was exploited by overexpressing *CKX3* under the control of the root-specific *PYK10* (hereafter designated *P10*) promoter. Plants expressing the *P10-CKX3* construct were crossed with four *IYG*-overproducing lines that enhance leaf growth (Gonzalez et al., 2009) and had been selected based on the reproducibility of their leaf phenotype and their functional diversity (Gonzalez et al., 2010). These IYGs encode the GROWTH REGULATING FACTOR 5 (GRF5), which is a putative transcription factor proposed to act in a pathway that promotes cell proliferation (Horiguchi et al., 2005); the GIBBERELLIN 20-OXIDASE 1 (GA20ox1) that catalyzes consecutive steps in the gibberellin biosynthesis (Xu et al., 1995); the BRASSINOSTEROID INSENSITIVE 1 (BRI1), a key component of the brassinosteroid membrane receptor complex (Wang et al., 2001); and the ARABIDOPSIS VACUOLAR PYROPHOSPHATASE 1 (AVP1), a H^+-pyrophosphatase involved in the generation of proton gradients in endomembrane compartments (Li et al., 2005). In all four lines, an increase in cell proliferation drives, predominantly or entirely, the enhanced leaf growth (Gonzalez et al., 2010).

Our results indicate that the phenotypic traits of the lines can be combined resulting in enhanced root and shoot growth. We provide evidence for crosstalk between cytokinins and brassinosteroids during lateral root and leaf growths and between cytokinins and gibberellins during leaf growth. In addition, a connection between cytokinins and *GRF5* during shoot growth is shown.

RESULTS

The *P10* promoter restricts the cytokinin-deficient phenotype of *CKX3* overexpression to the root

Overexpression of *CKX1* or *CKX3* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (*35S*) increases the total root system length of 8-day-old plants up to 3-fold, but drastically delays leaf formation and reduces leaf size (Werner et al., 2003). To benefit from the positive effect on root growth of *CKX* overexpression, while alleviating the inhibiting effect on shoot growth, the Arabidopsis *P10* promoter was chosen to drive

CKX3 expression. *PYK10* encodes a myrosinase and is highly expressed in hypocotyl and roots, while it is virtually not expressed in mature rosette leaves (Nitz et al., 2001; Sherameti et al., 2008). Plants with enhanced root growth and normal shoot growth could be obtained in this manner (Werner et al., 2010).

Root growth of *35S-CKX1* and *P10-CKX3-GFP* (further referred to as *P10-CKX3*) was measured to evaluate the potential of the *P10* promoter to confer the cytokinin-deficient root phenotype under our experimental conditions. In *35S-CKX1* plants, both primary root length (Fig. 1A) and lateral root number (Fig. 1B) had increased strongly compared with those of wild-type Columbia-0 (Col-0) 9 days after stratification (DAS). Primary root length and lateral root number of *P10-CKX3* plants were increased to the same extent as for *35S-CKX1* plants (Fig. 1, A and B), demonstrating that the *P10* promoter driving *CKX3* expression is sufficiently strong to confer the same root phenotype as the *35S* promoter driving *CKX1* expression.

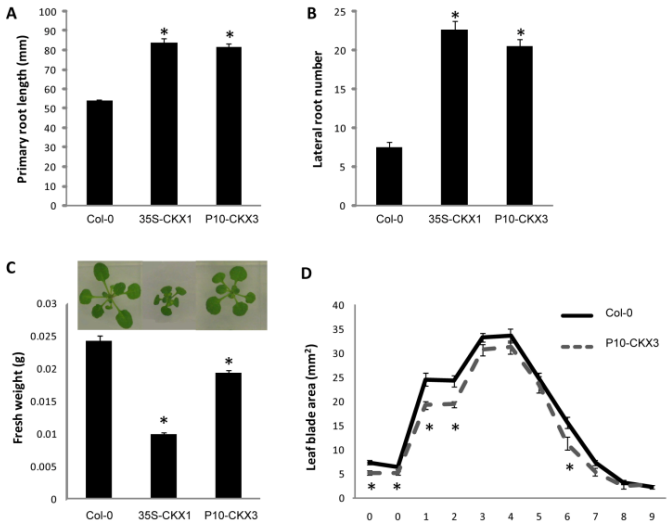


Figure 1. Enhanced root growth and slightly reduced shoot growth of *P10-CKX3* plants.

A and B, Primary root growth and lateral root number of *35S-CKX1* and *P10-CKX3* and wild-type (Col-0) lines. Plants were grown on vertical plates under in vitro conditions for 9 days. *, Significantly different from the wild type ($P < 0.01$, Student's *t* test). Error bars are SE ($n \geq 20$). C and D, Shoot growth. C, Fresh weight of rosettes of *35S-CKX1*, *P10-CKX3* and wild-type plants grown for 21 days under in vitro conditions. Pictures were taken from 18-day-old plants grown in vitro. Error bars are SE ($n \geq 21$). D, Blade area of cotyledons (0) and leaves 1 to 9 calculated from leaf series made at 21 DAS from *P10-CKX3* and wild-type plants grown in vitro. Error bars are SE ($n \geq 14$). *, Significantly different from the wild type ($P < 0.05$, Student's *t* test).

To quantify the effect of *CKX3* overexpression under the control of the *P10* promoter on shoot growth, fresh weight of the rosette was measured from plants grown for 21 DAS under in vitro conditions (Fig. 1C). This revealed that *P10-CKX3* plants show only a mild reduction in rosette size compared to wild-type plants, whereas constitutive overexpression of *CKX1* strongly reduced shoot growth (Werner et al., 2003) (Fig. 1C). To analyze the leaf size of *P10-CKX3* plants in detail, leaf parameters (blade area, length and width) of each individual leaf were calculated from leaf series made from 21-day-old rosettes (Fig. 1D and Supplemental Fig. 1). Although the blade area of *P10-CKX3* leaves was not significantly different from that of wild type for most leaves (Fig. 1D), leaf length was significantly reduced for all leaves, while blade width remained unchanged (Supplemental Fig. 1). This polarity-dependent reduction in blade area gave *P10-CKX3* plants a characteristic appearance with rounded laminas (Fig. 1C).

In conclusion, the use of the *P10* promoter to drive the expression of *CKX3* in Arabidopsis allows circumvention of the negative effect on shoot growth, but maintenance of the positive effect of *CKX* overexpression on root growth.

Crossing *P10-CKX3* plants with plants overexpressing genes that enhance leaf size

To assess whether genes that positively affect root growth can be combined with genes that enhance leaf organ size and whether this strategy could lead to a further increase in growth and biomass production, four different genes that enhance leaf size when overexpressed (*BRI1, GRF5, GA20ox1,* and *AVP1*) were co-overexpressed with the *P10-CKX3* construct.

Plants homozygous for each construct were crossed and root and shoot growths of the resulting heterozygous F1 progeny were analyzed. Each homozygous line was, in addition, crossed with wild-type Col-0 plants, resulting in heterozygous lines that were used as appropriate controls. To evaluate the effect on growth, the expected values in case of an additive effect were calculated as the sum of the measurements for each individual heterozygous parent minus the measurement for Col-0 for a certain trait. An additive phenotype corresponds to the sum of the individual phenotypes of the heterozygous parents and is, therefore, an indication that both genes might work independently to control organ size. When the combination of two transgenes results in a value for a phenotypic trait that

is higher or lower than the expected value for an additive effect, a putative synergistic or antagonistic growth phenotype occurs, respectively. The latter cases are usually an indication that the combined genes function in related pathways, making it possible to further unravel the regulatory network in which these genes take part.

Introduction of *pBRI-BRI1* into *P10-CKX3* plants results in a more than additive increase of root and shoot growth

Overexpression of *BRI1* under the control of its own promoter leads to an increased response to brassinosteroids resulting in longer petioles and larger leaves (Wang et al., 2001). However, when the plants were grown under in vitro conditions for 21 days, enhanced *BRI1* expression did not result in a significant increase neither in rosette fresh weight (Fig. 2A) nor in blade area for most leaves (Fig. 2B), but mainly in increased blade length (Supplemental Fig. 2, A and C). Heterozygous or homozygous *pBRI-BRI1-GFP* (further referred to as *BRI1*) plants were similarly affected (Fig. 2A). Also crossing with wild type had no effect on the extent of the reduced shoot growth in *P10-CKX3* lines (Fig. 2A). Rosette fresh weight of plants that overexpressed both *BRI1* and *CKX3* was equal to that of wild-type and *BRI1* plants (Fig. 2A), demonstrating that *BRI1* overexpression can compensate for the reduction in fresh weight caused by *CKX3* overexpression. Consequently, fresh weight of the cross was significantly higher than that of the expected value for an additive effect (Fig. 2A), as reflected in a synergistic increase in blade area for certain leaves (Fig. 2B). Detailed observation of leaf parameters showed that this was mainly attributed to an increase in leaf length that was comparable to that of Col-0×*BRI1* (Supplemental Fig. 2, B and D), suggesting that enhanced expression of *BRI1* overcomes the effects of *P10-CKX3* in the leaf length direction. Relative transgene expression levels in shoots of *BRI1*×*P10-CKX3* plants were similar to those of the heterozygous parents (Supplemental Fig. 3A), showing that the observed synergism in leaf blade area is not a consequence of an altered expression level of one transgene due to the presence of the other.

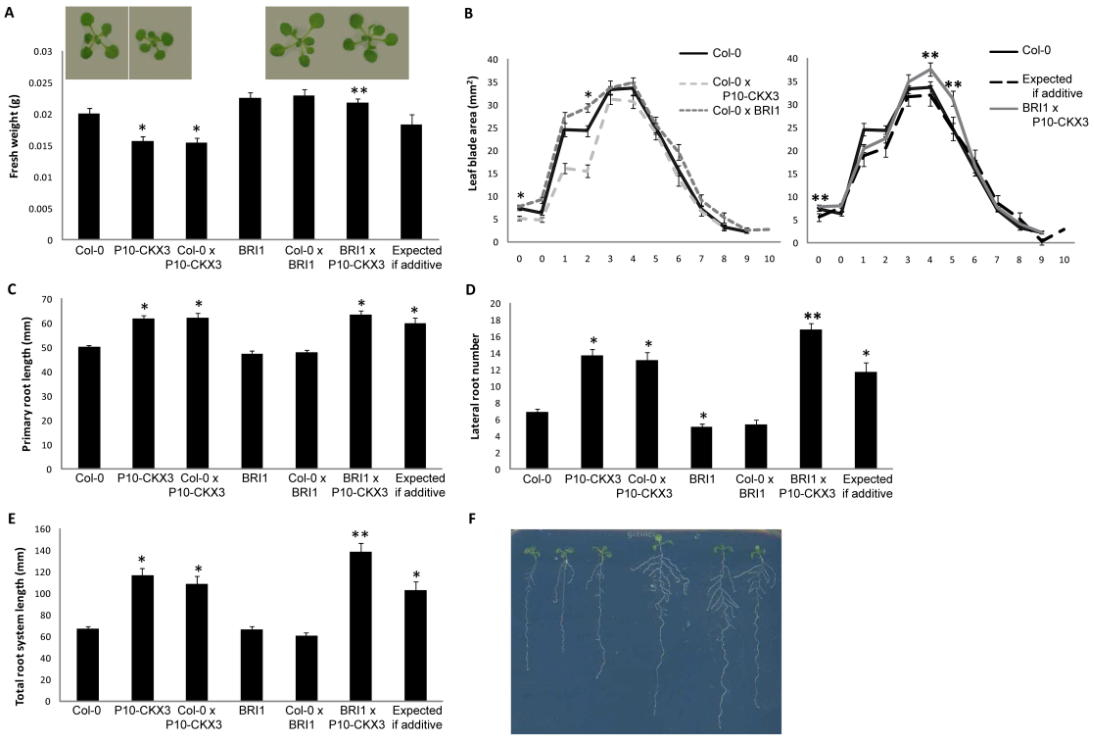


Figure 2. More than additive increases in shoot and root growth by crossing *P10-CKX3* plants with *BRI1* overexpressing plants.

A and B, Shoot phenotype of plants grown under in vitro conditions for 21 days. A, Rosette fresh weight. Pictures were taken from the rosettes of the wild type (Col-0), the heterozygous parents and the cross grown for 18 days in vitro. B, Blade area of cotyledons (0) and leaves 1 to 10 calculated from leaf series. C-F, Root growth of wild type plants and plants overexpressing *P10-CKX3* and/or *BRI1*. Plants were grown on vertical plates under in vitro conditions for 9 days. C, Primary root length. D, Lateral root number. E, Total root system length. F, Root system of 9-day-old plants. From left to right: Col-0, *BRI1*, Col-0×*BRI1*, *P10-CKX3*×*BRI1*, Col-0×*P10-CKX3*, and *P10-CKX3*. Values expected if additive were calculated as the sum of Col-0×*P10-CKX3* and *BRI1*×Col-0 minus Col-0. Asterisks indicate values significantly different from the wild type (*) and from the value expected for an additive effect (**) by Student's *t* test ($P < 0.05$ [A-B] and $P < 0.01$ [C-E]). Error bars are SE ($n \geq 16$ [A-B] and $n \geq 23$ [C-E]).

Whereas treatment with brassinolide (BL) has been shown to enhance lateral root formation (Bao et al., 2004), *BRI1*-overexpressing plants had no significantly enhanced root growth at 9 DAS (Fig. 2, C-F). No difference in root growth could be observed neither between homozygous and heterozygous *BRI1* lines nor between homozygous and heterozygous *P10-CKX3* lines (Fig. 2, C-F). Surprisingly, lateral root number and total root system length of *BRI1*×*P10-CKX3* were significantly larger than that expected for an

additive effect (Fig. 2, D and E). The expression level of both transgenes did not differ in the cross when compared to the heterozygous controls (Supplemental Fig. 3B). Although the lateral root number in Col-0×*P10-CKX3* plants was 90% higher than that in wild type, an increase of 144% was observed for plants overexpressing both *BRI1* and *CKX3* (Fig. 2D). Measurements of the total root system of Col-0×*P10-CKX3* revealed a 63% increase compared to control plants versus 107% in *BRI1*×*P10-CKX3* plants (Fig. 2E). The primary root was not significantly longer than the value expected for an additive effect (Fig. 2C). Hence, combining enhanced expression of these two genes has a synergistic effect on lateral root growth. Taken together, overexpression of *CKX3* and *BRI1* does not only result in the combination of phenotypic traits, but also improves lateral root growth synergistically and growth of particular leaves in more than an additive manner.

Exogenous application of brassinolide enhances lateral root growth of *P10-CKX3* plants

The observed positive effect of the combined overexpression of *CKX3* and *BRI1* suggests that reduced cytokinin levels and enhanced perception of BL act synergistically to enhance lateral root growth. To confirm the effect of brassinosteroids, *P10-CKX3* plants were treated with different concentrations of BL (Fig. 3). Wild-type primary root length decreased for all BL concentrations applied (Fig. 3A), as reported previously (Müssig et al., 2003; Bao et al., 2004). The relative decrease in primary root length was similar in *P10-CKX3* plants (Fig. 3A). Whereas no significant increase in lateral root number was observed when wild-type plants were treated with 1 to 100 nM BL under our conditions, lateral root number per cm of primary root of *P10-CKX3* plants increased in response to 1 to 50 nM BL (Fig. 3B). Moreover, the total length of *P10-CKX3* lateral roots increased up to 160% by treatment with 1 to 50 nM BL, whereas a significant increase up to 72% could only be detected for wild-type lateral roots for 1 and 2 nM BL (Fig. 3C). No correlation between the BL-induced lateral root phenotypes and changes in relative mRNA abundance of *CKX3* and *BRI1* in wild-type and *P10-CKX3* seedlings could be observed (Supplemental Fig. 3B). These data show that both lateral root formation and lateral root elongation are

further induced by the combined action of brassinosteroids and *CKX3* overexpression, and by inference a reduction of biologically active cytokinins.

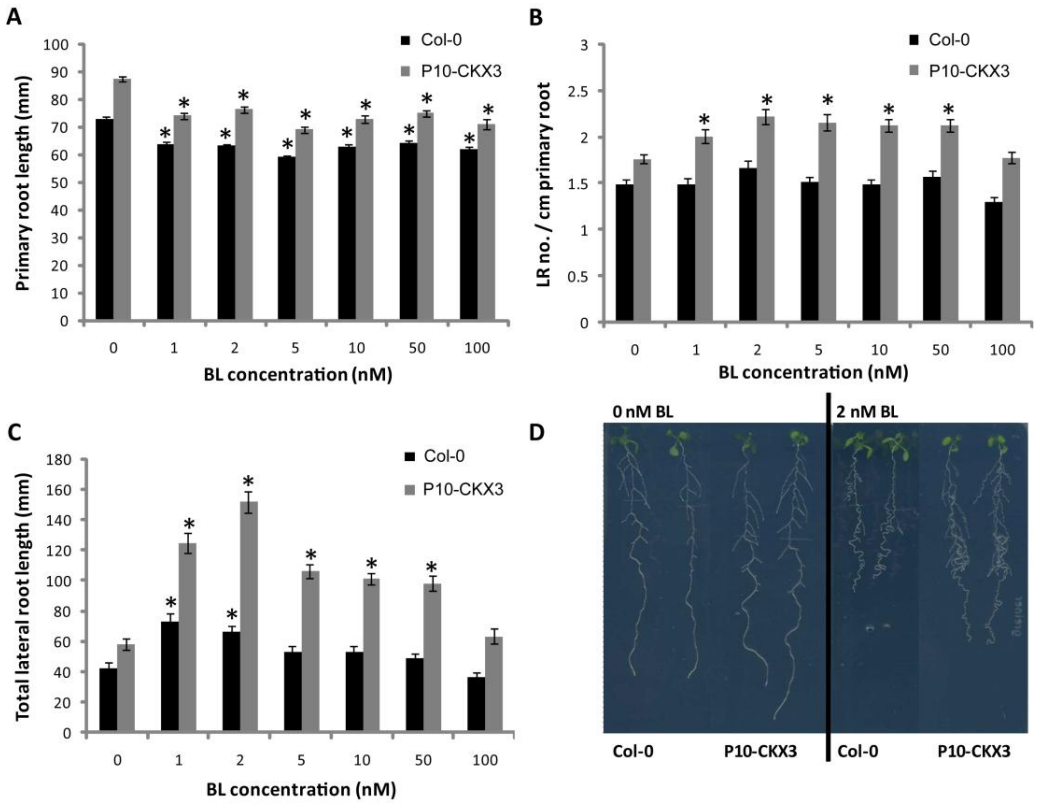


Figure 3. Synergistically enhanced lateral root growth of *P10-CKX3* plant by exogenous application of BL.

A-C, *P10-CKX3* and wild type (Col-0) seedlings grown vertically for 5 days on ½MS and transferred to plates containing ½MS + 0, 1, 2, 5, 10, 50, and 100 nM BL for 6 days. Primary root length (A), lateral root number per cm primary root (B), and total lateral root length (C) were determined. *, Significantly different from the mock-treated controls ($P < 0.01$, Student's *t* test). Error bars are SE ($n \geq 21$). D, Root system of 11-day-old plants, grown for 5 days on ½MS and 6 days on 0 (left) or 2 nM (right) BL.

Co-overexpression of *CKX3* with *GRF5* antagonizes the effects of *GRF5* during leaf development

Overexpression of *GRF5* leads to the development of enlarged leaves due to enhanced cell proliferation in both width and length directions (Horiguchi et al., 2005; Gonzalez et al., 2010), with an increase in rosette fresh weight of 21-day-old plants grown in vitro as a consequence (Fig. 4A). The cotyledons and first leaves were larger in size, but from leaf 3 onward, the blade area was slightly smaller than those of wild type (Fig. 4B) (Gonzalez et al., 2010). This characteristic leaf phenotype quantitatively differed between homozygous and heterozygous *GRF5*-overproducing lines, being less severe for heterozygous lines, indicating the presence of a gene dosage effect for *GRF5* (Fig. 4A). Crossing *P10-CKX3* with *GRF5* led to an average fresh weight that was slightly higher than that of wild type, but did not significantly differ from the expected value for an additive effect (Fig. 4A). Analysis of individual leaf blade areas however revealed that the increase in blade size caused by *GRF5* overexpression was reduced more than expected for an additive effect (Fig. 4B). In addition, the younger leaves 3 to 6, which are slightly smaller in both individual overexpressing lines, surprisingly were larger than expected if additive and even larger than those of wild-type leaves 3 and 4, when overexpression of *CKX3* and *GRF5* was combined (Fig. 4B). Suppression of the *CKX3* and *GRF5* phenotypes was observed in both width and length direction (Supplemental Fig. 2B and D). Analysis of relative mRNA levels of *CKX3* and *GRF5* eliminated the possibility that the effects resulted from changes in transgene expression (Supplemental Fig. 3D). A plausible explanation for the phenotype is that both genes antagonize each other: *CKX3* overexpression seems to reduce the effects of *GRF5* overexpression and/or vice versa, eventually converting a reduction in leaf size into an increase.

GRF5-overexpressing plants showed no significant enhanced root growth (Fig. 4, C and D). Root length and lateral root number in *GRF5*×*P10-CKX3* plants increased up to the same level as in *P10-CKX3* plants crossed with Col-0 (Fig. 4, C and D), indicating that *GRF5* overexpression does not influence the root phenotype caused by *CKX3* overexpression in the root.

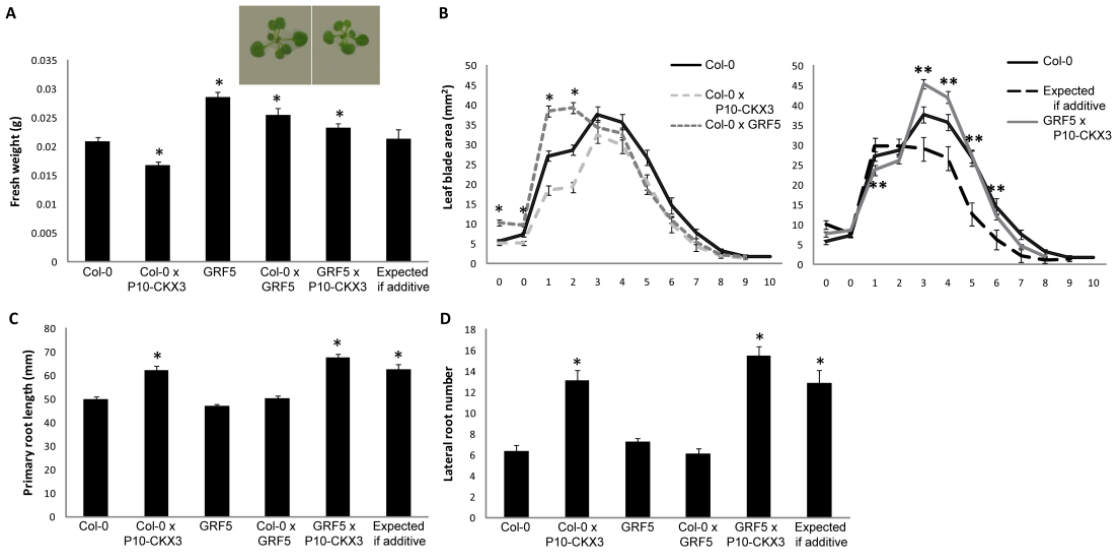


Figure 4. Leaf and root growth phenotypes of plants co-overexpressing *P10-CKX3* and *GRF5*.

A and B, Shoot growth measurements at 21 DAS from plants grown in vitro. A, Rosette fresh weight. Photographs were taken from the rosettes of the heterozygous parents and the cross grown for 18 days in vitro. B, Blade area of cotyledons (0) and leaves 1 to 10 calculated from leaf series. C-D, Root growth measurements of plants grown on vertical plates under in vitro conditions for 9 days. C, Primary root length. D, Lateral root number. Values expected if additive were calculated as the sum of the single heterozygous lines minus wild type (Col-0). Asterisks indicate values significantly different from the wild type (*) and from the value expected for an additive effect (**) by Student's *t* test ($P < 0.05$ [A-B] and $P < 0.01$ [C-D]). Error bars are SE ($n \geq 16$ [A-B] and $n \geq 23$ [C-D]).

The effects of *GA20ox1* and *CKX3* ectopic expression are synergistic with respect to leaf growth

Plants overexpressing *GA20ox1* under the control of the *35S* promoter show an increased content of bioactive GA4 and other GAs in the vegetative rosette (Coles et al., 1999; Gonzalez et al., 2010). These plants typically have elongated hypocotyls and stems and large leaves with long petioles (Coles et al., 1999). At 21 DAS, *35S-GA20ox1* (further referred to as *GA20ox1*) plants showed an increase in rosette fresh weight when compared to wild-type plants and the phenotype of heterozygous and homozygous plants was indistinguishable (Fig. 5A). Blade area of all rosette leaves had increased (Fig. 5B) as a consequence of enhanced growth in both blade width and length directions (Supplemental Fig. 2, A and C). Co-expression of *GA20ox1* and *P10-CKX3* resulted in a more than

additive effect on shoot growth (Fig. 5, A and B). *GA20ox1* overexpression not only compensated for the reduction in blade area due to *CKX3* overexpression, the blade area of *GA20ox1*×*P10-CKX3* plants additionally increased from leaf 3 onward compared to Col-0×*GA20ox1* plants (Fig. 5B) and this resulted from a further growth increase in the lateral direction (Supplemental Fig. 2, B and D). As blade width of *P10-CKX3* plants did not significantly change, *CKX3* overexpression might enhance the growth-promoting effect of *GA20ox1* overexpression in the width direction. This strong synergistic effect yielded up to a 36% increase in total rosette fresh weight compared to wild-type plants (Fig. 5A). Similar transgene mRNA levels in the cross and the heterozygous parents excluded that the observed synergism is caused by mutual induced changes in expression level (Supplemental Fig. 3E).

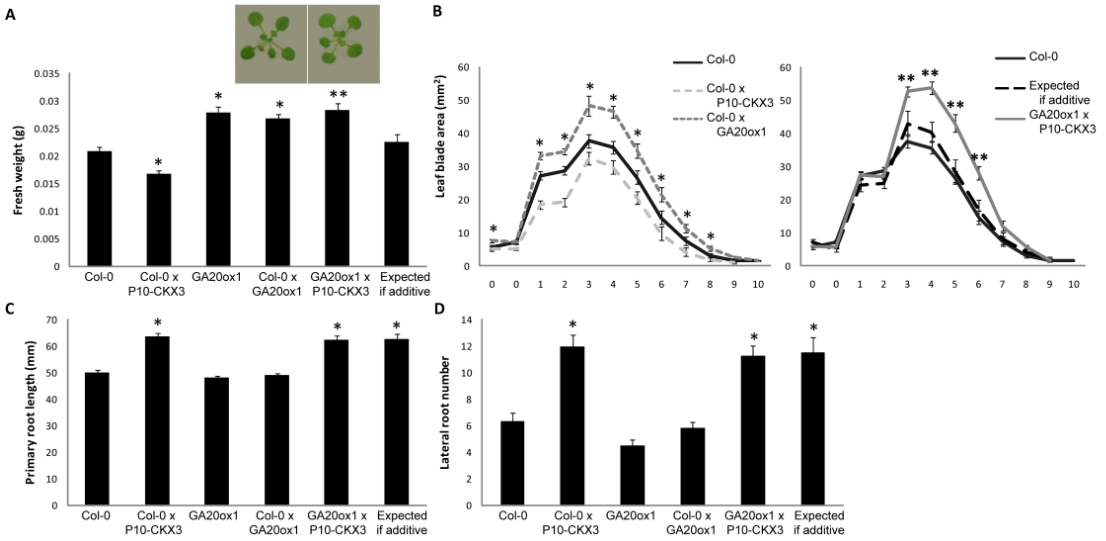


Figure 5. Synergistic and additive increase in leaf and root growth, respectively, by combined overexpression of *P10-CKX3* and *GA20ox1*.

A and B, Shoot phenotype of 21-day-old plants grown in vitro. A, Rosette fresh weight. Pictures were taken from the rosettes of the heterozygous parents and the cross grown for 18 days in vitro. B, Blade area of cotyledons (0) and leaves 1 to 10 calculated from leaf series. C-D, Root phentoype of plants grown on vertical plates under in vitro conditions for 9 days. C, Primary root length. D, Lateral root number. Values expected if additive were calculated as the sum of the single heterozygous lines minus wild type (Col-0). Asterisks indicate values significantly different from the wild type (*) and from the value expected for an additive effect (**) by Student's *t* test ($P < 0.05$ [A-B] and $P < 0.01$ [C-D]). Error bars are SE ($n \geq 16$ [A-B] and $n \geq 23$ [C-D]).

Overexpression of *GA20ox1* did not alter root growth, both in homozygous and heterozygous conditions (Fig. 5, C and D). Combination of *GA20ox1* with *P10-CKX3* led to an increase in total root system length characteristic for *CKX3* overexpression alone (Fig. 5, C and D), demonstrating that *GA20ox1* overexpression does not interfere with the effects of enhanced cytokinin breakdown in the root.

Combining *CKX3* and *AVP1* overexpression has an additive effect on root and shoot growth

AVP1 overexpression dramatically enhances leaf organ size by a polarity-independent increase in cell number (Li et al., 2005). However, homozygous *35S-AVP1* (further referred to as *AVP1*) plants exhibit only a slight increase in leaf organ size when grown under in vitro conditions on half-strength Murashige and Skoog (½MS) medium for 21 days (Gonzalez et al., 2010). Therefore, fresh weight and leaf parameters were analyzed from plants grown in soil (Fig. 6, A-C). All rosette leaves increased in size (Fig. 6C), leading to a doubling in rosette fresh weight when compared to wild-type plants, both for homozygous and heterozygous *AVP1* plants (Fig. 6A). Co-expressing *AVP1* and *P10-CKX3* led to an intermediate rosette growth, with measurements that equaled values expected for additive effects for fresh weight and all leaf parameters (Fig. 6, A and C; Supplemental Fig. 2, B and D). This results in a shoot phenotype at 22 DAS that more closely resembled wild-type shoot growth.

Also an increase in root growth was reported when plants were grown hydroponically for 45 days (Li et al., 2005). At 9 DAS however, root growth of homozygous *AVP1* plants was drastically reduced when grown in vitro on ½MS medium (Fig. 6, D and E). A striking difference in growth behavior between homozygous and heterozygous *AVP1*-overproducing plants could be observed, because primary root length and lateral root number of heterozygous *AVP1* plants were comparable to those of wild type (Fig. 6, D and E). Taken together, the analysis of in vitro root and shoot (data not shown) growth of heterozygous and homozygous *AVP1* plants suggests that the effect of ectopic expression of *AVP1* depends on the gene dosage. Root growth of *AVP1* plants crossed with *CKX3* plants was similar to that of Col-0×*P10-CKX3* plants, indicating that the combination of

both genes results in the phenotype of *P10-CKX3* plants (Fig. 6, D and E). In conclusion, these observations imply an independent mode of action of *AVP1* and *CKX3* through distinct pathways during root and shoot growth.

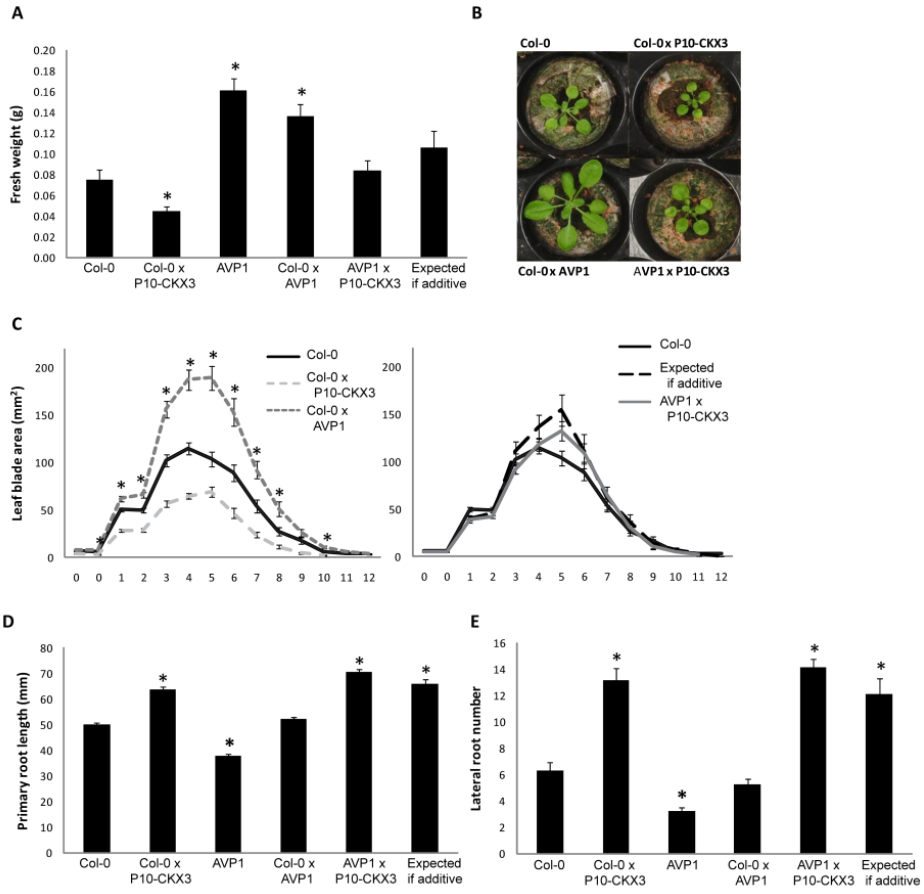


Figure 6. Additive effect on leaf and root growth by crossing of *P10-CKX3* and *AVP1* overexpressing plants.

A-C, Shoot growth of 22-day-old plants grown in soil. A, Rosette fresh weight. B, Pictures from the rosettes of wild-type (Col-0), the heterozygous parents and the cross. C, Blade area of cotyledons (0) and leaves 1 to 12 calculated from leaf series. D-E, Root phenotype of plants grown on vertical plates under in vitro conditions for 9 days. D, Primary root length. E, Lateral root number. Values expected for an additive effect were calculated as the sum of Col-0×*P10-CKX3* and Col-0×*AVP1* minus Col-0. Asterisks indicate values significantly different from the wild type (*) and from the value expected for an additive effect (**) by Student's *t* test ($P < 0.05$ [A-C] and $P < 0.01$ [D-E]). Error bars are SE ($n \geq 12$ [A-C] and $n \geq 23$ [D-E]).

DISCUSSION

Here, we show that the combined introduction of two transgenes, one enhancing root growth and the other enhancing shoot growth, is a powerful strategy to increase overall plant growth. The *P10* promoter driving *CKX3* expression proves to be efficient to confine cytokinin degradation to the root, thereby strongly reducing the negative effect of constitutive *CKX* overexpression on vegetative shoot growth (Werner et al., 2003). At 21 DAS, the leaf size of *P10-CKX3* plants is smaller than that of wild type, indicating a slightly slower development characteristic for plants with reduced cytokinin responses (Werner et al., 2003; Miyawaki et al., 2006; Riefler et al., 2006). This is most likely caused by a leaky expression from the *P10* promoter in the shoot early during development, since *CKX3* transcript levels are higher in *P10-CKX3* compared to wild-type rosettes, although they are much lower relative to root *CKX3* levels. However, rosette growth catches up with wild-type plants after bolting when plants are grown in soil (Werner et al., 2010) and expression of *P10-CKX3* has no significant influence on the leaf phenotypes obtained by simultaneous overexpression of *BRI1*, *GRF5*, *GA20ox1* or *AVP1* after bolting (data not shown). This implies that in the control of the whole plant size, root growth is seemingly not enhanced at the expense of enhanced shoot growth in the crosses, even in the absence of supplemented sucrose. Furthermore, it demonstrates that the increased root system does not act as a strong sink, which is in agreement with observations in tobacco (Werner et al., 2008) and Arabidopsis (Werner et al., 2010). Conversely, a fast-growing root system might provide more water and nutrients to the shoot, but no additional increase in shoot fresh weight was observed in comparison with the single *BRI1*-, *GRF5*-, *GA20ox1*- or *AVP1*-overexpressing lines, when root growth was enhanced by co-expression of *P10-CKX3*. Taken together, this strongly suggests that the phenotypic effects on root and shoot growth under optimal environmental conditions where assimilates are not limited, are primarily delineated by changes in genetic competence and not by the global sink-source regulation.

Unexpected phenotypic outcomes from crossing the transgenic lines were uncovered when leaf size and root architecture were analyzed at 21 and 9 DAS, respectively. These phenotypes that are not additive, are usually an indication for crosstalk between pathways

in which the genes function (Chandler, 2009; Pérez-Pérez et al., 2009) and will be discussed below.

Brassinosteroid–cytokinin crosstalk during leaf development

The reduction in leaf length caused by *CKX3* overexpression is completely overcome by simultaneous overexpression of *BRI1*. In other words, an increased sensitivity to brassinosteroids could compensate for the decreased leaf length resulting from enhanced cytokinin breakdown. Brassinosteroids were shown to affect both leaf cell proliferation and elongation, because the reduced leaf size of biosynthetic mutants is due to a decrease in cell number and size (Nakaya et al., 2002). However, overexpression of *BRI1* enhances cell proliferation in the leaf, while no changes in cell size were detected (Gonzalez et al., 2010). Moreover, the changes in leaf size caused by *BRI1* overexpression might be the consequence of an increase in the period rather than the rate of cell proliferation (Gonzalez et al., 2010). A role for cytokinins in the duration of leaf cell proliferation has also been suggested through the maintenance of meristematic competence to prevent the transition to differentiation and, hence, cell expansion (Nishimura et al., 2004). Taken together these observations suggest crosstalk between pathways that guide brassinosteroid and cytokinin responses toward cell division mechanisms, thereby controlling the timing of transition from cell proliferation to cell expansion.

Brassinosteroids can increase cell division, in particular when auxin and cytokinin are limiting and it was demonstrated that BL can substitute cytokinin to promote growth of callus and cell suspensions of Arabidopsis (Hu et al., 2000). In good agreement with our findings, an increased brassinosteroid response compensates for lower cytokinin levels in the leaves. Little is known however on the molecular mechanisms that integrate the responses from both hormones. Hormonal crosstalk can be accomplished by the regulation of expression of common target genes or modulation of the activity of a common protein, which is considered as direct crosstalk (Chandler, 2009). Expression of *Cyclin D3;1* (*CYCD3;1*), a positive regulator of cell proliferation, is induced by treatment with brassinosteroids and cytokinins (Riou-Khamlichi et al., 1999; Hu et al., 2000), making it a good candidate. Numerous other genes have been found to be transcriptionally regulated by

both hormones (Nemhauser et al., 2006; Peng et al., 2009), but their putative role as convergence point of brassinosteroid and cytokinin response pathways needs further investigation.

Alternatively, hormonal crosstalk can be indirect, when one hormone response pathway influences the biosynthesis, degradation, sensitivity, or perception of the other hormone (Chandler, 2009). Given that enhanced *BRI1* expression compensates for lower cytokinin levels in the leaf, under normal conditions, cytokinins might potentially attenuate the brassinosteroid response in one of the above described ways. Genes involved in brassinosteroid metabolism are likely targets, although changes in their expression induced by cytokinin treatment were not solely linked to decreased brassinosteroid levels (Nemhauser et al., 2006).

Brassinosteroid–cytokinin crosstalk during lateral root development

Root growth is impaired in brassinosteroid biosynthetic mutants and in the signaling mutant *bri1*, demonstrating that brassinosteroids positively affect root growth (Müssig et al., 2003; Bao et al., 2004; Mouchel et al., 2006). On the other hand, neither overexpression of *BRI1* nor BL treatment increase primary root growth or lateral root formation of wild-type plants. In fact, BL treatment reduces primary root growth of wild-type and *P10-CKX3* plants to the same relative extent. Also, primary root growth of *P10-CKX3* plants is not influenced by simultaneous *BRI1* overexpression. Although changes in brassinosteroid as well as cytokinin levels can affect the size of the root apical meristem (Werner et al., 2003; Mouchel et al., 2006; Dello Ioio et al., 2007), both hormones seemingly accomplish that through independent, parallel pathways.

In contrast to previous observations (Bao et al., 2004), BL has no effect on the formation of lateral roots of wild-type plants. However, when cytokinin levels are reduced in the root, BL concentrations from 1 to 50 nM enhance lateral root formation and elongation. Also combining *CKX3* overexpression with *BRI1* overexpression enhances lateral root growth synergistically, demonstrating that an enhanced sensitivity to brassinosteroids as well as exogenous application of brassinosteroids further stimulates lateral root growth of *P10-CKX3* plants. In other words, cytokinin deficiency enhances the effect of brassinosteroids

on lateral root growth or vice versa. Therefore we hypothesize that under normal conditions, cytokinins antagonize brassinosteroid function in lateral root formation and/or vice versa.

Whereas virtually nothing is known on the molecular basis of cytokinin–brassinosteroid crosstalk during lateral root development, ample evidence exists for the interaction of both hormones with auxin. Brassinosteroids promote the initiation of lateral root primordia, most probably by increasing acropetal auxin transport to aid local auxin accumulation necessary for primordium development (Benková et al., 2003; Bao et al., 2004). They are proposed to act through modulation of *PINFORMED* (*PIN*) auxin efflux carriers, because brassinosteroids have been shown to regulate *PIN* gene expression (Nakamura et al., 2004; Nemhauser et al., 2004). Cytokinins inhibit lateral root initiation by preventing re-entry of pericycle cells into the cell cycle, thereby perturbing asymmetric cell division to establish lateral root primordia (Li et al., 2006). In addition, cytokinins disturb auxin accumulation by downregulation of *PIN* gene expression, inhibiting cell fate respecification in the developing primordium (Laplaze et al., 2007), suggesting that crosstalk between both hormones is accomplished through modulation of auxin transport. Furthermore, both brassinosteroids and cytokinins regulate the transcription of genes involved in the auxin signaling pathway (Nakamura et al., 2003, 2006; Goda et al., 2004; Mouchel et al., 2006; Nemhauser et al., 2006; Hardtke, 2007; Dello Ioio et al., 2008).

Alternatively the observed synergism can be brought about by direct crosstalk between cytokinin and brassinosteroid response pathways. *BREVIS RADIX* (*BRX*) is putatively involved, because the mutant, which has a root-specific brassinosteroid deficiency (Mouchel et al., 2004), is insensitive for inhibition of lateral root formation when cytokinins are applied, due to an altered auxin response (Li et al., 2009), underlining the complexity of the highly interconnected hormonal network that governs plant growth.

Gibberellin–cytokinin crosstalk during leaf development

Overexpression of *CKX3* does not interfere with the increase in blade length resulting from *GA20ox1* overexpression and synergistically enhances the increase in blade width. This observation demonstrates that increased gibberellin levels overcome the reduction in blade length caused by enhanced cytokinin breakdown and reduced cytokinin levels enhance the growth promoting effect of gibberellins during growth in the blade width direction. Thus, cytokinins and gibberellins interact antagonistically to promote leaf growth, consistent with their interaction during other plant developmental processes (Greenboim-Wainberg et al., 2005; Jasinski et al., 2005; Weiss and Ori, 2007).

Overexpression of *GA20ox1* considerably enhances leaf cell number, whereas cell size is only moderately increased (Gonzalez et al., 2010). Gibberellins promote growth through the proteolytical degradation of growth-repressing DELLA proteins that is likewise documented to stimulate both cell elongation and proliferation (Achard et al., 2009). Cytokinin deficiency or insensitivity affects cell number, suggesting that crosstalk between cytokinins and gibberellins during leaf growth concerns cell division (Werner et al., 2003; Nishimura et al., 2004). Consistently with this hypothesis, CYCD3;1 plays an important role in executing cytokinin (Dewitte et al., 2007) as well as gibberellin responses, because overexpression of *CYCD3;1* can rescue the growth of gibberellin-deficient *ga1-3* plants (Achard et al., 2009).

Evidence for crosstalk between cytokinin and gibberellin response pathways exists at both the level of biosynthesis regulation and signal transduction. Two gibberellin biosynthesis genes, *GA20ox* and *GA4*, are downregulated, while two *DELLA* genes, *GAI* and *RGA*, are upregulated upon cytokinin treatment of seedlings (Brenner et al., 2005). The gibberellin-response inhibitor SPINDLY (SPY) is proposed to mediate crosstalk between cytokinin and gibberellin responses (Greenboim-Wainberg et al., 2005; Weiss and Ori, 2007). Whereas SPY promotes cytokinin responses, it represses gibberellin responses, while gibberellins inhibit cytokinin signaling by suppression of SPY activity through a DELLA-independent pathway (Maymon et al., 2009). Whether SPY integrates gibberellin and cytokinin responses to control cell division and/or cell expansion mechanisms during leaf growth remains to be elucidated.

GRF5–cytokinin crosstalk during leaf development

Co-expression of *CKX3* and *GRF5* suppresses leaf phenotypes of the individual overexpressing parent plants, giving rise to blade areas that more closely resemble those of wild type. This implies that when cytokinin levels are lowered, the effects of *GRF5* overexpression disappear and vice versa, suggesting that cytokinins and *GRF5* work in concert to stimulate leaf cell proliferation. Like cytokinins, *GRF5* is postulated to regulate the transition between cell proliferation and expansion (Gonzalez et al., 2010). Expression of this putative transcription factor coincides with actively dividing cells and rapidly declines when expansion starts (Horiguchi et al., 2005, Andriankaja et al., 2012).

Transcript profiling of *GRF5*-overexpressing seedlings did not reveal any significant enrichment for genes involved in cytokinin responses (Gonzalez et al., 2010) and *GRF5* was not transcriptionally regulated by cytokinin treatment or *CKX1* overexpression (Brenner et al., 2005; Nemhauser et al., 2006; Lee et al., 2007; Argyros et al., 2008), suggesting that *GRF5* does not function directly in the cytokinin signaling pathway nor influences cytokinin metabolism. Therefore, crosstalk between *GRF5* and *CKX3*, and by inference cytokinins, might rather be accomplished by the regulation of (a) common target gene(s) or protein(s). Genes found to be differentially regulated by overexpression of *GRF5* (Gonzalez et al., 2010) and in the same direction by cytokinin treatment (Argyros et al., 2008) and/or in the opposite direction by *CKX1* overexpression (Brenner et al., 2005), are putative candidates for the convergence of both response pathways.

Interestingly, the leaves of *GRF5* plants are dark green due to an increased chlorophyll content (L. Vercruyssen, V.B. Tognetti, N. Gonzalez, F. Van Breusegem and D. Inzé, unpublished data). Cytokinins are known to stimulate chlorophyll synthesis and to increase the photosynthetic rate (Wareing et al., 1968; Mok, 1994; Riefler et al., 2006; Argyros et al., 2008), illustrating that *GRF5* and cytokinin functions are most probably interconnected during leaf development.

AVP1

AVP1 is implicated in polar auxin transport (Li et al., 2005) and overexpression of *AVP1* increases auxin content in seedlings (Gonzalez et al., 2010). Although several lines of evidence exist for cytokinin-auxin crosstalk (Bögre et al., 2008; Benková and Hejátko, 2009; Chandler, 2009; Veit, 2009), co-expression of *AVP1* and *CKX3* results in root and leaf phenotypes corresponding to the sum of the phenotypes of the individual heterozygous parent plants, at least under our growth conditions. Therefore, *AVP1* and *CKX3* seem to function in independent pathways. Alternatively, because the leaf size of *AVP1*×*P10-CKX3* plants closely resembles that of wild type, the effects of increased auxin levels can be counteracted by those of reduced cytokinin levels, resulting in a restored auxin/cytokinin balance that favors wild-type cell proliferation timing.

Conclusion

Diverse hormonal inputs are guided through different, but interconnected, response pathways that form complex networks and determine the final extent of cell proliferation and cell expansion in roots and leaves. By simultaneously altering the expression of two functionally distinct components in the network, we reveal crosstalk and bring insight into the hierarchies among the distinct pathways. Further study is needed to identify the "nodes" of the network, the molecular players that integrate the information from these different pathways to control final organ size. On the other hand, root and shoot growth appear to be less tightly linked and it seems likely from our experiments that the effects on cell proliferation and/or expansion are superimposed to changes in the sink-source balance in the whole plant, at least under optimal conditions.

Enhancing root growth can provide several advantages, such as a better exploitation of soil nutrients and water when environmental conditions are less favorable (Nibau et al., 2008) and is becoming a key feature of ameliorating crop productivity (Gewin, 2010). Simultaneously increasing the size of leaves, which provide the major energy for the plant, might alter the photosynthetic efficiency and create the potential for further biomass improvement under diverse environmental conditions (Zhu et al., 2010). Furthermore,

many examples illustrate that the above-described mechanisms that control plant growth and, additionally, tolerance to abiotic stress, in Arabidopsis can be readily translated to improve crop productivity. For instance, overexpression of *OsGA20ox1* greatly enhances plant stature of rice (*Oryza sativa*) (Oikawa et al., 2004) and homologs of *BRI1* and *CKX* genes are present in many crop species, where they perform similar functions (Yamamuro et al., 2000; Chono et al., 2003; Schmülling et al., 2003). Increased tolerance to salt or drought stresses observed in *AVP1*-overexpressing plants (Gaxiola et al., 2001) has been conducted in tobacco, creeping bentgrass (*Agrostis stolonifera*), and tomato (*Solanum lycopersicum*) by heterologous expression (Park et al., 2005; Duan et al., 2007; Li et al., 2010). This suggests that combining these transgenes will most probably result in the combination of beneficial phenotypic traits in crops.

ACKNOWLEDGEMENTS

We sincerely thank the colleagues in the Systems Biology of Yield group for fruitful discussions, Véronique Storme for the statistical analysis of the qRT-PCR data, and Martine De Cock for help in preparing the manuscript.

MATERIALS AND METHODS

Plant material

Seeds of the *Arabidopsis thaliana* (L.) Heyhn. were from the *35S-CKX1* (*CKX1*; At2G41510) and *P10-CKX3* (*CKX3*; At5G56970) lines (Werner et al., 2003; Werner et al., 2010); those of the *AVP1*-overexpressing line (At1G15690), the *GRF5*-overexpressing line (At3G13960), the *BRI1*-overexpressing line (At4G39400), and the *GA20ox1*-overexpressing line (At4G25420) were kindly provided by Dr. Roberto A. Gaxiola (University of Connecticut) (Li et al., 2005), Dr. Hirokazu Tsukaya (University of Tokyo) (Horiguchi et al., 2005), Dr. Joanne Chory (The Salk institute, California) (Wang et al., 2001), and Dr. Peter Hedden (Rothamsted Research, UK) (Coles et al., 1999), respectively. Seeds from all the transgenic lines were in Columbia-0 (Col-0) background.

Growth conditions

As environmental conditions during seed formation and seed storage duration can affect seed vigor, all experiments were conducted with wild-type and transgenic seeds that had been harvested from plants grown side by side on the same tray. For in vitro experiments, seeds were sown in sterile plates containing half-strength Murashige and Skoog (½MS) medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.8% agar. The plates were sealed and put in a tissue culture room at 21°C under a 16-h day/8-h night regime for shoot growth analysis and under continuous light (110 μmol m^{-2} s^{-1}) for root growth analysis. *AVP1*-overexpressing plants were grown in soil for shoot growth analysis at 22°C under 16-h day/8-h night conditions (50 μmol m^{-2} s^{-1}). Brassinolide (BL) was purchased from Fuji Chemical Industries (Toyama, Japan), dissolved in DMSO, and added to the agar medium after autoclaving. Plants were grown under 16-h day/8-h light conditions for 5 days on ½ MS prior to transfer to ½ MS medium containing different concentrations of BL for another 6 days.

Growth analysis

To measure rosette leaf parameters, seedlings were grown under in vitro conditions for 21 days (*AVP1*-overexpressing plants in soil for 22 days). Individual leaves (cotyledons and

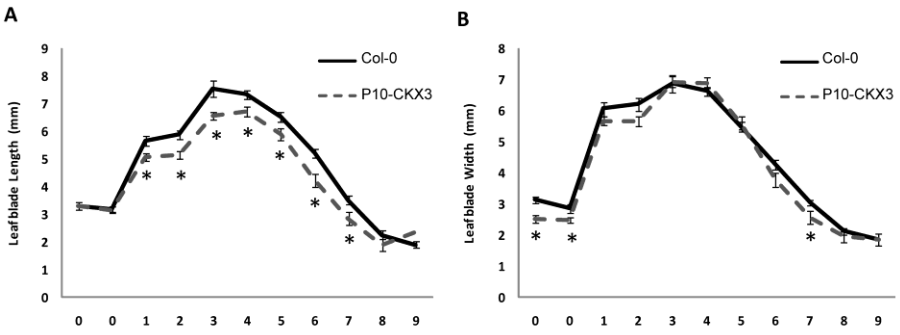
rosette leaves) were dissected and photographed. Their blade area, blade width, and blade length were measured with the ImageJ software (http://rsb.info.nih.gov/ij/).

For root system analysis, seedlings were grown on vertical plates for 9 or 11 (BL treatments) days, after which the plates were scanned (Epson). Primary root length, lateral root length, and total root system length were measured with the ImageJ software (http://rsb.info.nih.gov/ij/).

Quantitative reverse-transcription (qRT)-PCR

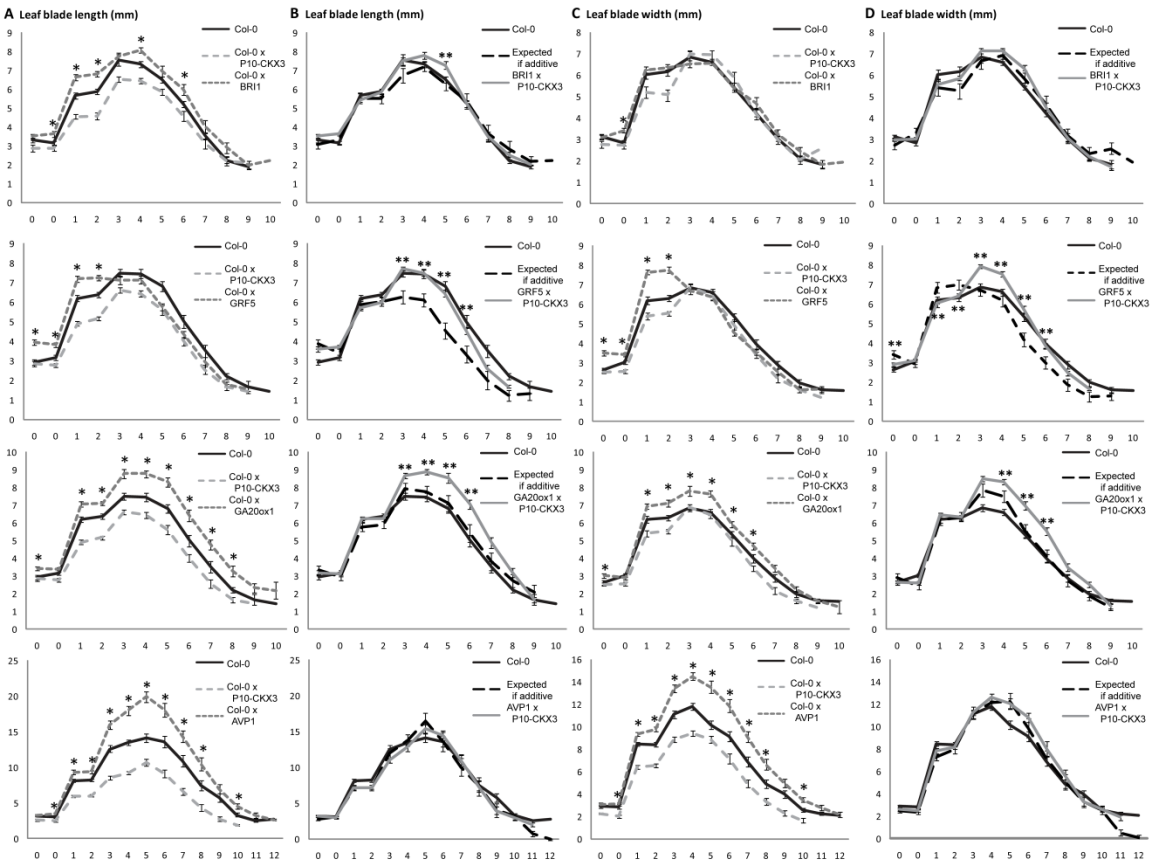
RNA was extracted according to a combined protocol of TRI Reagent RT (Molecular Research Center) and the RNeasy kit (QIAGEN) with on-column DNase (QIAGEN) digestion. cDNA was prepared from 250 ng to 1 µg of RNA with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Following primer pairs were used: 5'-TCACTAGCGGTCCTGTTCTTG-3' and 5'-TCAAAAGCCTCCCAATTGTC-3' for *CKX3*, 5'-AAAGTTGCGGTTGCGTGTTTG-3' and 5'-GTTGACTGTGAATCTATCCCTGACC-3' for *BRI1*, 5'-TCAGTTCAATGTCTTAGCCTCTGC-3' and 5'-CCCAACTCCTCCAACTCTCTCC-3' for *GRF5* and 5'-CATCAACGTTCTCGAGCTTGATGTTC-3' and 5'-GCGGCTCGTGTATTCATGAGCG-3' for *GA20ox1*. qRT-PCR was done on a LightCycler 480 (Roche Diagnostics) on 384-well plates with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's recommendations. All individual reactions were done in triplicate. Expression levels were normalized against the average of the housekeeping genes *UBQ10*, *CKA2*, and *CDKA1;1* and relative expression levels of the crosses were determined with the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) or qBase (Hellemans et al., 2007). Normalized values for the biological replicates of BL treated samples were used for statistical analysis according to the proc mixed procedure from SAS (SAS 9.2, Cary, NC: SAS Institute) including plate as random factor. Least square mean differences with mixed-model based *t* tests and p-values were calculated between 0 nM BL and each BL concentration and that for each line.

SUPPLEMENTAL DATA



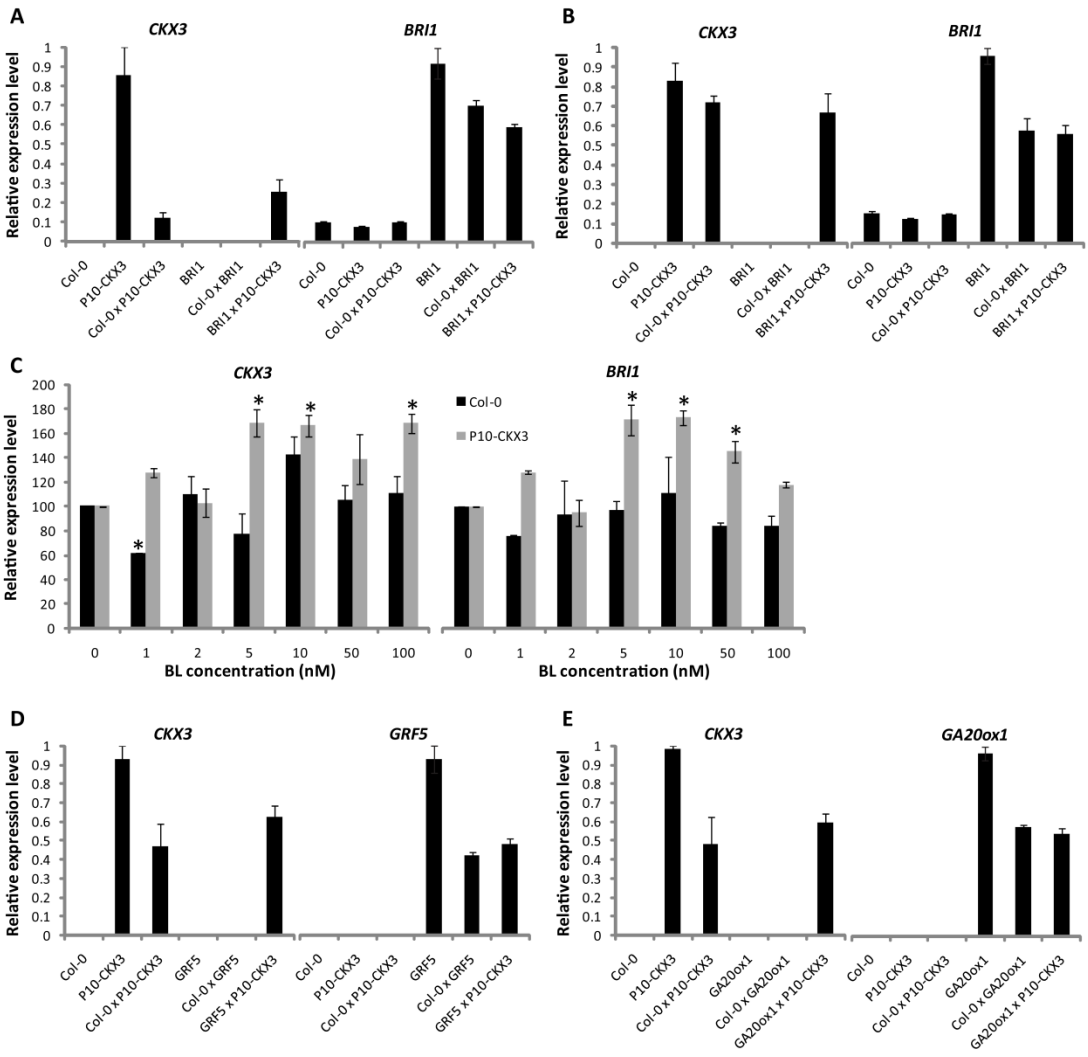
Supplemental Figure S1.

Leaf parameters for wild-type (Col-0) and *P10-CKX3* plants calculated from leaf series made at 21 DAS from plants grown in vitro. A, Blade length. B, Blade width of cotyledons (0) and leaves 1 to 9. *, Significantly different from the wild type ($P < 0.05$, Student's *t* test). Error bars are SE ($n \geq 14$).



Supplemental Figure S2.

Leaf parameters of *P10-CKX3* plants crossed with *BRI1*, *GRF5*, *GA20ox1* and *AVP1*-overexpressing plants, respectively, for cotyledons (0) and leaves 1 to 10 or 12. A and B, Blade length. C and D, Blade width. Blade length and width were calculated from leaf series made at 21 DAS from plants grown in vitro or at 22 DAS from plants grown in soil (*AVP1*×*P10-CKX3*). Blade length (A) and width (C) of wild type and the heterozygous parents. Blade length (B) and width (D) of wild type and the crosses and the expected values for an additive effect. Values expected if additive were calculated as the sum of the single heterozygous lines minus wild type. * and **, Significantly different from the wild type and the value expected for an additive effect, respectively ($P < 0.05$, Student's *t* test). Error bars are SE ($n \geq 14$).



Supplemental Figure S3.

Relative mRNA levels of *CKX3*, *BRI1*, *GRF5* and *GA20ox1* determined by qRT-PCR. A and B, Expression levels of *CKX3* and *BRI1* in wild type (Col-0), *P10-CKX3*, *BRI1*, *BRI1*×*P10-CKX3* and the heterozygous control plants in (A) the shoot of plants at 14 DAS and (B) in roots of 9-day-old seedlings. C, *CKX3* and *BRI1* Expression levels in wild-type and *P10-CKX3* plants treated with 1 to 100 nM brassinolide (BL) relative to mock-treated controls (0 nM BL) for each line at 11 DAS. *, Significantly different from mock-treated control ($P < 0.05$, Mixed-model based *t* test). D and E, Relative expression levels in wild type seedlings, homo- and heterozygous parents and crossed seedlings at 5 DAS. D, *CKX3* and *GRF5* expression. E. *CKX3* and *GA20ox1* expression. Error bars are SE (n = 6 for crosses or n = 2).

REFERENCES

Andriankaja M, Dhondt S, De Bodt S, Vanhaeren H, Coppens F, De Milde L, Mühlenbock P, Skirycz A, Gonzalez N, Beemster GTS, Inzé D (2012) Exit from proliferation during leaf development in *Arabidopsis thaliana*: a not-so-gradual process. Developmental Cell **22:** 64-78

Achard P, Gusti A, Cheminant S, Alioua M, Dhondt S, Coppens F, Beemster GTS, Genschik P (2009) Gibberellin signaling controls cell proliferation in *Arabidopsis*. Curr Biol **19:** 1188-1193

Argueso CT, Ferreira FJ, Kieber JJ (2009) Environmental perception avenues: the interaction of cytokinin and environmental response pathways. Plant Cell Environ **32:** 1147-1160

Argyros RD, Mathews DE, Chiang Y-H, Palmer CM, Thibault DM, Etheridge N, Argyros DA, Mason MG, Kieber JJ, Schaller GE (2008) Type B response regulators of *Arabidopsis* play key roles in cytokinin signaling and plant development. Plant Cell **20:** 2102-2116

Bao F, Shen J, Brady SR, Muday GK, Asami T, Yang Z (2004) Brassinosteroids interact with auxin to promote lateral root development in Arabidopsis. Plant Physiol **134:** 1624-1631

Beemster GTS, De Veylder L, Vercruysse S, West G, Rombaut D, Van Hummelen P, Galichet A, Gruissem W, Inzé D, Vuylsteke M (2005) Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. Plant Physiol **138:** 734-743

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115:** 591-602

Benková E, Hejátko J (2009) Hormone interactions at the root apical meristem. Plant Mol Biol **69:** 383-396

Bögre L, Magyar Z, López-Juez E (2008) New clues to organ size control in plants. Genome Biol **9:** 226

Borlaug N (2007) Feeding a hungry world. Science **318:** 359-359

Brenner WG, Romanov GA, Köllmer I, Bürkle L, Schmülling T (2005) Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J **44:** 314-333

Chandler JW (2009) Auxin as compère in plant hormone crosstalk. Planta **231:** 1-12

Chono M, Honda I, Zeniya H, Yoneyama K, Saisho D, Takeda K, Takatsuto S, Hoshino T, Watanabe Y (2003) A semidwarf phenotype of barley uzu results from a nucleotide substitution in the gene encoding a putative brassinosteroid receptor. Plant Physiol **133:** 1209–1219

Coles JP, Phillips AL, Croker SJ, García-Lepe R, Lewis MJ, Hedden P (1999) Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes. Plant J **17:** 547-556

Dello Ioio R, Nakamura K, Moubayidin L, Perilli S, Taniguchi M, Morita MT, Aoyama T, Costantino P, Sabatini S (2008) A genetic framework for the control of cell division and differentiation in the root meristem. Science **322:** 1380-1384

Dello Ioio R, Scaglia Linhares F, Scacchi E, Casamitjana-Martinez E, Heidstra R, Costantino P, Sabatini S (2007) Cytokinins determine *Arabidopsis* root-meristem size by controlling cell differentiation. Curr Biol **17:** 678-682

Dewitte W, Scofield S, Alcasabas AA, Maughan SC, Menges M, Braun N, Collins C, Nieuwland J, Prinsen E, Sundaresan V, Murray JAH (2007) *Arabidopsis* CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proc Natl Acad Sci USA **104:** 14537-14542

Donnelly PM, Bonetta D, Tsukaya H, Dengler RE, Dengler NG (1999) Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. Dev Biol **215:** 407-419

Duan X-G, Yang A-F, Gao F, Zhang S-L, Zhang J-R (2007) Heterologous expression of vacuolar H^+-PPase enhances the electrochemical gradient across the vacuolar membrane and improves tobacco cell salt tolerance. Protoplasma **232:** 87-95

Eathington SR, Crosbie TM, Edwards MD, Reiter RS, Bull JK (2007) Molecular markers in a commercial breeding program. Crop Sci **47:** S154-S163

Edgerton MD (2009) Increasing crop productivity to meet global needs for feed, food, and fuel. Plant Physiol **149:** 7-13

Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. Proc Natl Acad Sci USA **98:** 11444-11449

Gewin V (2010) An underground revolution. Nature **466:** 552-553

Goda H, Sawa S, Asami T, Fujioka S, Shimada Y, Yoshida S (2004) Comprehensive comparison of auxin-regulated and brassinosteroid-regulated genes in Arabidopsis. Plant Physiol **134:** 1555-1573

Gonzalez N, Beemster GTS, Inzé D (2009) David and Goliath: what can the tiny weed *Arabidopsis* teach us to improve biomass production in crops? Curr Opin Plant Biol **12:** 157-164

Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhont S, Van Daele T, De Milde L, Weigel D, Kamiya Y, Stitt M, Beemster GTS, Inzé D (2010) Increased leaf size: different means to an end. Plant Physiol **153:** 1261-1279

Greenboim-Wainberg Y, Maymon I, Borochov R, Alvarez J, Olszewski N, Ori N, Eshed Y, Weiss D (2005) Cross talk between gibberellin and cytokinin: the Arabidopsis GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. Plant Cell **17:** 92-102

Guivarc'h A, Rembur J, Goetz M, Roitsch T, Noin M, Schmülling T, Chriqui D (2002) Local expression of the *ipt* gene in transgenic tobacco (*Nicotiana tabacum* L. cv. SR1) axillary buds establishes a role for cytokinins in tuberization and sink formation. J Exp Bot **53:** 621-629

Hardtke CS (2007) Transcriptional auxin-brassinosteroid crosstalk: who's talking? BioEssays **29:** 1115-1123

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol **8:** R19.1-R19.14

Horiguchi G, Kim G-T, Tsukaya H (2005) The transcription factor AtGRF5 and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of *Arabidopsis thaliana*. Plant J **43:** 68-78

Hu Y, Bao F, Li J (2000) Promotive effect of brassinosteroids on cell division involves a distinct *CycD3*-induction pathway in *Arabidopsis*. Plant J **24:** 693-701

James C (2009) Global Status of Commercialized Biotech/GM Crops: 2009, ISAAA Brief No 41. The International Service for the Acquisition of Agri-biotech Applications (ISAAA), Ithaca, NY

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P, Tsiantis M (2005) KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. Curr Biol **15:** 1560-1565

Krizek BA (2009) Making bigger plants: key regulators of final organ size. Curr Opin Plant Biol **12:** 17-22

Kudo T, Kiba T, Sakakibara H (2010) Metabolism and long-distance translocation of cytokinins. J Integr Plant Biol **52:** 53-60

Kuiper D (1993) Sink strength: established and regulated by plant growth regulators. Plant Cell Environ **16:** 1025-1026

Laplaze L, Benkova E, Casimiro I, Maes L, Vanneste S, Swarup R, Weijers D, Calvo V, Parizot B, Herrera-Rodriguez MB, Offringa R, Graham N, Doumas P, Friml J, Bogusz D, Beeckman T, Bennett M (2007) Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell **19:** 3889-3900

Lee DJ, Park J-Y, Ku S-J, Ha Y-M, Kim S, Kim MD, Oh M-H, Kim J (2007) Genome-wide expression profiling of *ARABIDOPSIS RESPONSE REGULATOR 7*(*ARR7*) overexpression in cytokinin response. Mol Genet Genomics **277:** 115-137

Li J, Mo X, Wang J, Chen N, Fan H, Dai C, Wu P (2009) BREVIS RADIX is involved in cytokinin-mediated inhibition of lateral root initiation in *Arabidopsis*. Planta **229:** 593-603

Li J, Yang H, Peer WA, Richter G, Blakeslee J, Bandyopadhyay A, Titapiwantakun B, Undurraga S, Khodakovskaya M, Richards EL, Krizek B, Murphy AS, Gilroy S, Gaxiola R (2005) *Arabidopsis* H^+-PPase AVP1 regulates auxin-mediated organ development. Science **310:** 121-125

Li X, Mo X, Shou H, Wu P (2006) Cytokinin-mediated cell cycling arrest of pericycle founder cells in lateral root initiation of *Arabidopsis*. Plant Cell Physiol **47:** 1112-1123

Li Z, Baldwin CM, Hu Q, Liu H, Luo H (2010) Heterologous expression of *Arabidopsis* H^+-pyrophosphatase enhances salt tolerance in transgenic creeping bentgrass (*Agrostis stolonifera* L.). Plant Cell Environ **33:** 272-289

Livak KJ, and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:** 402-408

Maymon I, Greenboim-Wainberg Y, Sagiv S, Kieber JJ, Moshelion M, Olszewski N, Weiss D (2009) Cytosolic activity of SPINDLY implies the existence of a DELLA-independent gibberellin-response pathway. Plant J **58:** 979-988

Miyawaki K, Tarkowski P, Matsumoto-Kitano M, Kato T, Sato S, Tarkowska D, Tabata S, Sandberg G, Kakimoto T (2006) Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proc Natl Acad Sci USA **103:** 16598-16603

Mok MC (1994) Cytokinins and plant development – an overview. In DWS. Mok, MC Mok, eds, Cytokinins: Chemistry, Activity, and Function. CRC Press, Boca Raton, FL, pp 155-166

Mok DWS, Mok MC (2001) Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol **52:** 89-118

Mouchel CF, Briggs GC, Hardtke CS (2004) Natural genetic variation in *Arabidopsis* identifies *BREVIS RADIX*, a novel regulator of cell proliferation and elongation in the root. Genes Dev **18:** 700-714

Mouchel CF, Osmont KS, Hardtke CS (2006) *BRX* mediates feedback between brassinosteroid levels and auxin signalling in root growth. Nature **443:** 458-461

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant **15:** 473-497

Müssig C, Shin G-H, Altmann T (2003) Brassinosteroids promote root growth in Arabidopsis. Plant Physiol **133:** 1261-1271

Nakamura A, Goda H, Shimada Y, Yoshida S (2004) Brassinosteroid selectively regulates *PIN* gene expression in Arabidopsis. Biosci Biotechnol Biochem **68:** 952-954

Nakamura A, Higuchi K, Goda H, Fujiwara MT, Sawa S, Koshiba T, Shimada Y, Yoshida S (2003) Brassinolide induces *IAA5*, *IAA19*, and DR5, a synthetic auxin response element in Arabidopsis, implying a cross talk point of brassinosteroid and auxin signaling. Plant Physiol **133:** 1843-1853

Nakamura A, Nakajima N, Goda H, Shimada Y, Hayashi K-i, Nozaki H, Asami T, Yoshida S, Fujioka S (2006) Arabidopsis *Aux/IAA* genes are involved in brassinosteroid-mediated growth responses in a manner dependent on organ type. Plant J **45:** 193-205

Nakaya M, Tsukaya H, Murakami N, Kato M (2002) Brassinosteroids control the proliferation in leaf cells of *Arabidopsis thaliana*. Plant Cell Physiol **43:** 239-244

Naqvi S, Farré G, Sanahuja G, Capell T, Zhu C, Christou P (2010) When more is better: multigene engineering in plants. Trends Plant Sci **15:** 48-56

Nemhauser JL, Hong F, Chory J (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell **126:** 467-475

Nemhauser JL, Mockler TC, Chory J (2004) Interdependency of brassinosteroid and auxin signaling in *Arabidopsis*. PLoS Biol **2:** e258 (1460-1471)

Nibau C, Gibbs DJ, Coates JC (2008) Branching out in new directions: the control of root architecture by lateral root formation. New Phytol **179:** 595-614

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. Plant Cell **16:** 1365-1377

Nitz I, Berkefeld H, Puzio PS, Grundler FMW (2001) *Pyk10*: a seedling and root specific gene and promoter from *Arabidopsis thaliana*. Plant Sci **161:** 337-346

Oikawa T, Koshioka M, Kojima K, Yoshida H, Kawata M (2004) A role of *OsGA20ox1*, encoding an isoform of gibberellin 20-oxidase, for regulation of plant stature in rice. Plant Mol Biol **55:** 687-700

Park S, Li J, Pittman JK, Berkowitz GA, Yang H, Undurraga S, Morris J, Hirschi KD, Gaxiola RA (2005) Up-regulation of a H^+-pyrophosphatase (H^+-PPase) as a strategy to engineer drought-resistant crop plants. Proc Natl Acad Sci USA **102:** 18830-18835

Paul MJ, Foyer CH (2001) Sink regulation of photosynthesis. J Exp Bot **52:** 1383-1400

Peng Z-y, Zhou X, Li L, Yu X, Li H, Jiang Z, Cao G, Bai M, Wang X, Jiang C, Lu H, Hou X, Qu L, Wang Z, Zuo J, Fu X, Su Z, Li S, Guo H (2009) Arabidopsis Hormone Database: a comprehensive genetic and phenotypic information database for plant hormone research in Arabidopsis. Nucleic Acids Res **37:** D975-D982

Pérez-Pérez JM, Candela H, Micol JL (2009) Understanding synergy in genetic interactions. Trends Genet **25:** 368-376

Riefler M, Novak O, Strnad M, Schmülling T (2006) *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18:** 40-54

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH (1999) Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. Science **283:** 1541-1544

Schmülling T, Werner T, Riefler M, Krupková E, Bartrina y Manns I (2003) Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, *Arabidopsis* and other species. J Plant Res **116:** 241-252

Sherameti I, Venus Y, Drzewiecki C, Tripathi S, Dan VM, Nitz I, Varma A, Grundler FM, Oelmüller R (2008) PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J **54:** 428-439

Veit B (2009) Hormone mediated regulation of the shoot apical meristem. Plant Mol Biol **69:** 397-408

Wang Z-Y, Seto H, Fujioka S, Yoshida S, Chory J (2001) BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature **410:** 380-383

Wareing PF, Khalifa MM, Treharne KJ (1968) Rate-limiting processes in photosynthesis at saturating light intensities. Nature **220:** 453-457

Weiss D, Ori N (2007) Mechanisms of cross talk between gibberellin and other hormones. Plant Physiol **144:** 1240-1246

Werner T, Holst K, Pörs Y, Guivarc'h A, Mustroph A, Chriqui D, Grimm B, Schmülling T (2008) Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. J Exp Bot **59:** 2659-2672

Werner T, Köllmer I, Bartrina I, Holst K, Schmülling T (2006) New insights into the biology of cytokinin degradation. Plant Biol **8:** 371-381

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15:** 2532-2550

Werner T, Nehnevajova E, Köllmer I, Novák O, Strnad M, Krämer U, Schmülling T (2010) Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in *Arabidopsis* and tobacco. Plant Cell *in press* (doi: 10.1105/tpc.109.072694)

Xu Y-L, Li L, Wu K, Peeters AJM, Gage DA, Zeevaart JAD (1995) The *GA5* locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: molecular cloning and functional expression. Proc Natl Acad Sci USA **92:** 6640-6644

Yamamuro C, Ihara Y, Wu X, Noguchi T, Fujioka S, Takatsuto S, Ashikari M, Kitano H, Matsuoka M (2000) Loss of function of a rice *brassinosteroid insensitive1* homolog prevents internode elongation and bending of the lamina joint. Plant Cell **12:** 1591-1605

Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science **287:** 303-305

Zhu X-G, Long SP, Ort DR (2010) Improving photosynthetic efficiency for greater yield. Annu Rev Plant Biol **61:** 235-261

3

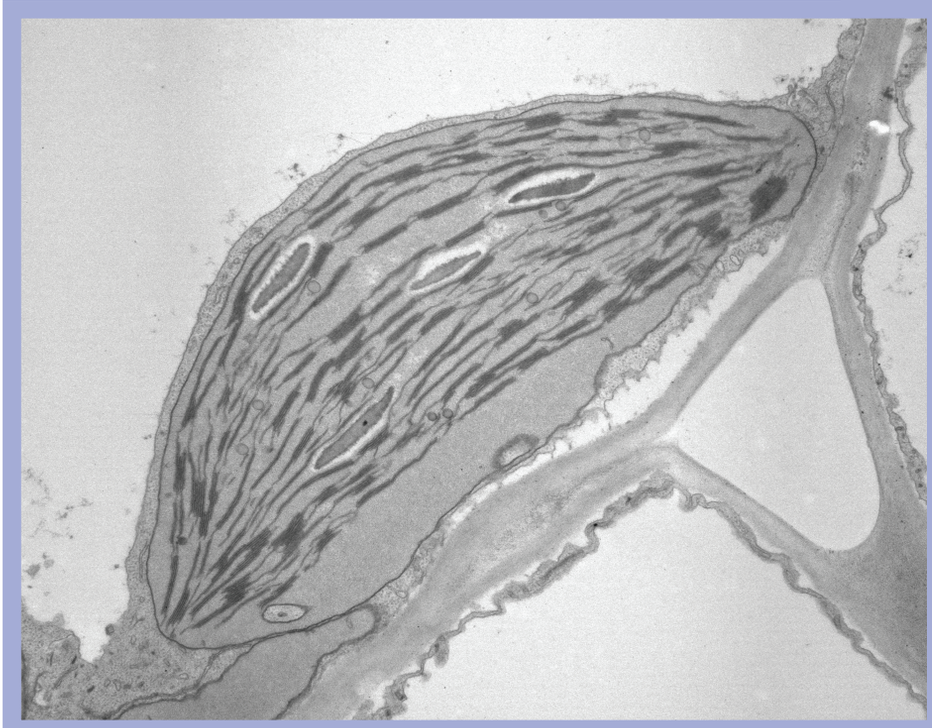


Image: Electron micrograph of a chloroplast from *35S:GRF5* Arabidopsis Col-0 leaves at 21 days after stratification.

Chapter 3: GROWTH REGULATING FACTOR 5 stimulates Arabidopsis chloroplast division and tolerance to nitrogen deprivation: cross talk with cytokinin signaling

Liesbeth Vercruyssen[1,2], Vanesa B. Tognetti[1,2,3], Riet De Rycke[1,2], Nathalie Gonzalez[1,2], Frank Van Breusegem[1,2], and Dirk Inzé[1,2]

[1]Department of Plant Systems Biology, VIB, 9052 Ghent, Belgium

[2]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium

[3]Current address: Central European Institute of Technolgoy (CEITEC), 60177 Brno, Czech Republic

L.V. wrote the manuscript and contributed, the cytokinin treatments and flowering observations. V.B.T. performed the photosynthetic measurements, the determination of ferredoxin levels and nitrogen deprivation assays. L.V. and V.B.T contributed the chloroplast data. R.D.R. performed the scanning electron microscopy. N.G., V.B.T., F.V.B. and D.I. contributed valuable comments, fruitful discussions and manuscript corrections.

ABSTRACT

Arabidopsis leaf development relies on subsequent phases of cell proliferation and cell expansion. During the proliferation phase, chloroplasts need to divide extensively and concomitant with the transition from cell proliferation to expansion, they differentiate into photosynthetically active chloroplasts, providing the plant with energy. The transcription factor GROWTH REGULATING FACTOR 5 (GRF5) functions to extend the cell proliferation period during leaf development. Cytokinins also promote leaf growth in part by extending the cell proliferation phase, simultaneously delaying the onset of the cell expansion phase. In addition, cytokinins are known to be involved in chloroplast development and nitrogen signaling. Here, it is shown that GRF5 promotes chloroplast division, resulting in a higher chloroplast number per cell with a concomitant increase in chlorophyll and ferredoxin levels in *35S:GRF5* leaves, which can sustain higher maximum rates of photosynthesis. Moreover, the increased chloroplast number and CO_2 assimilation most likely render *35S:GRF5* plants more tolerant for growth on nitrogen depleted medium. Evidence is provided that GRF5 and cytokinins

synergistically enhance cell division, which makes suspect that they also cooperate to stimulate chloroplast division and/or nitrogen assimilation. Taken together with the increased leaf size, ectopic expression of *GRF5* has great potential to improve crop productivity under diverse environmental conditions.

INTRODUCTION

Arabidopsis leaves initiate as primordia at the flank of the shoot apical meristem (SAM) by extensive cell divisions. Later during leaf development, cell proliferation ceases with the arrest of the mitotic cell cycle, and cell expansion starts, concomitant with the onset of endoreduplication, i.e. genome replication without cell division (Donnelly et al., 1999; Beemster et al., 2005). The mitotic arrest front where cells transition from proliferation to expansion, initiates quickly in the distal part of the leaf, remains at an almost constant relative position for a few days and ultimately disappears abruptly near the leaf base (Kazama et al., 2010; Andriankaja et al., 2012). *GROWTH REGULATING FACTOR 5* (*GRF5*) encodes a transcription factor that promotes cell proliferation during leaf development, by delaying the exit from the cell proliferation phase (Horiguchi et al., 2005; Gonzalez et al., 2010). GRF5 functions partially redundant with eight other members of the GRF family, and forms a complex with the transcriptional coactivator GRF INTERACTING FACTOR 1/ANGUSTIFOLIA 3 (GIF1/AN3), to regulate target gene transcription (Horiguchi et al., 2005; Kim and Lee, 2006). The onset of cell expansion is tightly linked with chloroplast differentiation, as demonstrated by transcript profiling during the transition from cell proliferation to cell expansion, which revealed an enrichment for genes involved in photosynthesis and chloroplast retrograde signaling (Andriankaja et al., 2012). More interestingly, retrograde signaling from the chloroplast and hence chloroplast differentiation was demonstrated to be a prerequisite for the correct timing of the start of cell expansion (Andriankaja et al., 2012).

It is since long known that application of kinetin, a synthetic cytokinin, enhanced the photosynthetic rate, measured as CO_2 assimilation, and stimulated callus greening and redifferentiation into shoot tissue (Miller et al., 1955; Skoog and Miller, 1957; Wareing et al., 1968). Cytokinins are well known now to promote seed germination, shoot apical

meristem establishment, leaf blade expansion, vascular tissue development and chloroplast development, while negatively affecting apical dominance and senescence (Mok, 1994; Hwang et al., 2012).

Cytokinin signaling is mediated by a two-component phosphorelay that initiates with the autophosphorylation of the receptor ARABIDOPSIS HISTIDINE KINASES (AHKs) upon cytokinin perception, followed by phosphotransfer to ARABIDOPSIS HIS PHOSPHOTRANSFER proteins (AHPs) and ending with the phosphorylation of ARABIDOPSIS RESPONSE REGULATORS (ARRs), of which two types can be distinguished: the A-type and B-type ARRs (Hwang and Sheen, 2001; Hutchison et al., 2006; Dortay et al., 2008; Hwang et al., 2012). The B-type ARRs function as transcription factors that induce the expression of the primary cytokinin response genes, including the A-type *ARRs* (Mason et al., 2005; Kim et al., 2006; Taniguchi et al., 2007; Argyros et al., 2008; Brenner et al., 2012). The latter are negative feedback regulators of cytokinin signaling (Kiba et al., 2003; To et al., 2004; Dortay et al., 2006; Lee et al., 2008). Moreover, several CYTOKININ RESPONSE FACTORS (CRFs) were identified as immediate-early cytokinin response targets, that interact with the AHPs and in turn regulate transcription of a large portion of cytokinin-response genes, many of them which are also differentially regulated by B-type ARRs (Rashotte et al., 2006; Cutcliffe et al., 2011; Brenner et al., 2012).

In addition to the components of the signaling pathway, cytokinin biosynthetic ISOPENTENYLTRANSFERASES (IPTs) and CYTOCHROME P450 monooxygenases (CYP735As), catabolic CYTOKININ OXIDASES/DEHYDROGENASES (CKXs), conjugating enzymes and activating LONELY GUY (LOG) enzymes contribute to the final cytokinin response by affecting the active cytokinin levels in the different plant tissues (Kakimoto, 2001; Takei et al., 2001; Werner et al., 2003; Takei et al., 2004b; Miyawaki et al., 2006; Kuroha et al., 2009; Kudo et al., 2010; Tokunaga et al., 2012).

The generation of mutants with compromised cytokinin metabolism or signaling confirmed the positive function of cytokinins in chloroplast development, thereby establishing the involvement of the two-component phosphorelay. Mutations of single *ahk2* and *ahk3*, double *ahk2/3* and triple *ahk2/3/4* cytokinin receptors progressively reduced chlorophyll content (Riefler et al., 2006; Argyros et al., 2008). Chlorophyll

levels were also decreased in the shoot of the B-type *arr1/10/12* triple mutant, affected in the majority of cytokinin-activated responses during vegetative plant development (Argyros et al., 2008; Ishida et al., 2008). In addition, the cotyledons of triple *crf1/2/5* mutants displayed reduced greening (Rashotte et al., 2006). On the other hand, overexpression of A-type *ARR16*, diminished leaf chlorophyll levels (Ren et al., 2009).

Chlorophyll retention upon dark-induced senescence and the regeneration of green shoot tissue from callus at different cytokinin concentrations, are frequently used assays to test transgenic plants for their sensitivity towards exogenous cytokinin application. For example, in detached and dark-incubated wild type leaves, loss of chlorophyll is delayed with increasing cytokinin concentrations, while the *ahk2/3/4* receptor mutant lost the ability to retain chlorophyll, indicating a strongly reduced cytokinin sensitivity (Riefler et al., 2006). Whereas the *arr1/10/12* mutant never formed green tissue with the range of cytokinin concentrations applied, the hextuple A-type *arr3/4/5/6/8/9* mutant showed enhanced shoot regeneration from callus, demonstrating their role as positive and negative regulators of cytokinin signaling, respectively (To et al., 2004; Mason et al., 2005). Consistent with that, overexpression of *LOG* genes, and ectopic expression of an undegradable form of B-type *ARR2*, enhanced chlorophyll retention and shoot regeneration, while overexpression of A-type *ARR7* resulted in the opposite effects (Lee et al., 2007; Kuroha et al., 2009; Kim et al., 2012).

Chloroplasts are inherited as proplastids, usually from the mother plant, and reside in meristematic tissue to differentiate into photosynthetically active chloroplasts in the leaves (Sakamoto et al., 2008). Light triggers the differentiation of etioplasts in the cotyledons of dark-grown seedlings, constituting a part of the photomorphogenesis response which is generally promoted by cytokinin (Chory et al., 1994; Vandenbussche et al., 2007). The molecular basis likely involves the stimulation of expression of two *GATA* transcription factor encoding genes, *GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED* (*GNC*) and *GNC-LIKE/CYTOKININ-RESPONSIVE GATA FACTOR 1* (*GNL/CGA1*), by light and cytokinins (Brenner et al., 2005; Kiba et al., 2005; Naito et al., 2007). GNC and GNL positively regulate chloroplast division, chlorophyll accumulation and the transcript and protein levels of PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (POR) enzymes, that catalyze the second last step of chlorophyll

production (Reinbothe et al., 1996; Oosawa et al., 2000; Su et al., 2001; Richter et al., 2010; Hudson et al., 2011; Kollmer et al., 2011). Cytokinins also stimulate the accumulation of the chlorophyll precursor protochlorophyllide (Pchlide) in the dark, since Pchlide levels are increased and reduced, respectively, in the *ckx* quadruple and the *ahk2/ahk3* double mutant (Hedtke et al., 2012). Accumulation of Pchlide occurs in the dark and is thought to allow for the rapid initiation of photosynthesis upon de-etiolation (Schoefs and Franck, 2003). Furthermore, cytokinin application enhances expression of chloroplast encoded genes as well as nuclear genes encoding chloroplast constituents, like the small subunit of rubisco (RBCS) (Chory et al., 1994; Mok, 1994; Brenner et al., 2005; Boonman et al., 2007; Zubo et al., 2008). In contrast, overexpression of *CKX1* in tobacco plants, led to premature differentiation of chloroplasts in the SAM, while leaf cell chloroplasts displayed signs of early senescence (Werner et al., 2008). However, this could be an indirect consequence of the role of cytokinins in promoting SAM maintenance and the duration of leaf cell proliferation while delaying the onset of cell differentiation (Holst et al., 2011; Hwang et al., 2012).

Nevertheless, chloroplasts not only need to differentiate when mesophyll cells develop from meristematic tissue, they also need to divide extensively. Cytokinins were shown to promote the division of chloroplasts and this could be, at least in part, mediated through *CRF2*. Overexpression of *CRF2*, as well as cytokinin treatment increased PLASTID DIVISION 2 (PDV2) levels, resulting in more, but slightly smaller chloroplast in leaves and cotyledons, suggesting an accelerated chloroplast division rate (Okazaki et al., 2009).

Furthermore, together with chloroplast development, the leaves gain the ability to assimilate nitrate by the chloroplast-driven activation of NITRATE REDUCTASE (NIA) in the cytosol (Lillo, 2008). Cytokinins function as secondary messengers that signal nitrogen availability, since nitrate is well-known to induce the expression of *IPT3* and *CYP735A2* biosynthetic enzymes (Miyawaki et al., 2004; Takei et al., 2004a). As such, cytokinins coordinate the acquisition of this essential nutrient from the soil with the amount required for growth, resulting in a balanced nutrient to growth status (Sakakibara et al., 2006; Argueso et al., 2009; Kiba et al., 2011). Cytokinins are postulated to negatively feed-back on nitrogen uptake by downregulation of nitrate transporters (*NRTs*) (Brenner et al., 2005; Kiba et al., 2005). However, cytokinin-stimulated upregulation of

shoot *NRTs*, *NIA* and other genes encoding enzymes involved in NH_4^+ assimilation, demonstrated that cytokinin signaling has the potential to increase nitrogen assimilation (Brenner et al., 2005; Kiba et al., 2011). Since *GNC* and *GNL* expression is also induced by nitrogen sources, the two transcription factors are proposed to integrate light, nitrogen and cytokinin signals, besides gibberellic acid (GA) signals, to regulate nitrogen assimilation and chloroplast development (Scheible et al., 2004; Bi et al., 2005; Hudson et al., 2011).

Previously, combined ectopic expression of *GRF5* with *CKX3* revealed that the *35S:GRF5*-driven increase in leaf size was suppressed by enhanced cytokinin degradation. Therefore, it was hypothesized that GRF5 and cytokinins work in concert to stimulate cell proliferation during leaf development (Vercruyssen et al., 2011). Interestingly, the leaves of *GRF5* overexpressing plants show more intense greening, suggesting an increase in chlorophyll levels, a phenotypic trait that could be caused by altered cytokinin signaling. By the analysis of chloroplasts and photosynthetic parameters, it is demonstrated here that overexpression of *GRF5* stimulates the division of chloroplasts, resulting in enhanced CO_2 assimilation. The latter is most likely responsible for the observed tolerance of *GRF5* overexpressing plants for growth on nitrogen depleted medium. Furthermore, evidence is provided that cytokinins increase the ability of GRF5 to promote cell proliferation in a developing leaf.

RESULTS

GRF5 stimulates chloroplast development

Besides being larger (Horiguchi et al., 2005; Gonzalez et al., 2010), a remarkable feature of the leaves of Arabidopsis plants constitutively overexpressing *GRF5* (*35S:GRF5*) is their darker green color compared to wild-type leaves, that can be observed when the plants are grown both *in vitro* and *in soil* (Fig. 1, A). Therefore, photosynthetic pigment concentration was determined in leaves of 21-day-old plants, revealing a strong increase in chlorophyll a (Chla) and Chlb as well as carotenoids, per cm^2 leaf surface in *35S:GRF5* plants, while the Chla/Chlb ratio was not altered compared to wild-type plants

(Fig. 1, B). Different processes can be the cause of the enhanced chloroplastic pigment levels, such as an increase in leaf thickness due to more or larger mesophyll cells, a larger number of chloroplasts per cell, an increase in chloroplast size, or an elevated pigment biosynthesis per chloroplast.

To distinguish between these possibilities, transverse sections were made of wild-type and *35S:GRF5* leaves 1&2, harvested at 21 days after stratification (DAS) of soil-grown plants. Leaf epidermal and subepidermal palisade cell numbers were shown previously to be strongly increased at this stage, whereas cell size was moderately reduced or unchanged, when measured from paradermal views (Horiguchi et al., 2005; Gonzalez et al., 2010). Measurements of the area of 200 wild-type and 200 *35S:GRF5* transverse sectioned palisade mesophyll cells adjacent to the adaxial epidermis, and determination of the corresponding chloroplast number, revealed that, despite the small increase in average cell size, *35S:GRF5* cells equal in size to wild-type cells contained more chloroplasts (Fig. 1, C). Subdivision of palisade mesophyll cell areas in different categories of a multiple of 1000 μm^2, and calculation of the average chloroplast number per category showed significant increases in *35S:GRF5* chloroplast numbers in palisade mesophyll cells area categories ranging from 1000 to 5000 μm^2 (Fig. 1, D). As a result, the average chloroplast number per transverse two-dimensional (2D) cell area was significantly increased (Fig. 1, E). No obvious differences in leaf thickness and organization of cell layers or chloroplast size could be observed between *GRF5* overexpressing and wild-type leaves (data not shown). Recent advances in 3D imaging of fully expanded Arabidopsis leaf 6, revealed that a large variation in cell areas corresponds to a large range in cell volumes in epidermal and mesophyll tissues, and for palisade mesophyll cells, the 2D findings can be extrapolated to 3D cell volumes by on average a minimum factor 25 and maximum factor 50 (Wuyts et al., 2010; Nathalie Wuyts, personal communication). Thus, the observed average 20% increase in 2D chloroplast number corresponds to a 3D increase of 500%-1000%, or in other words, *35S:GRF5* cells contain five to ten times more chloroplasts.

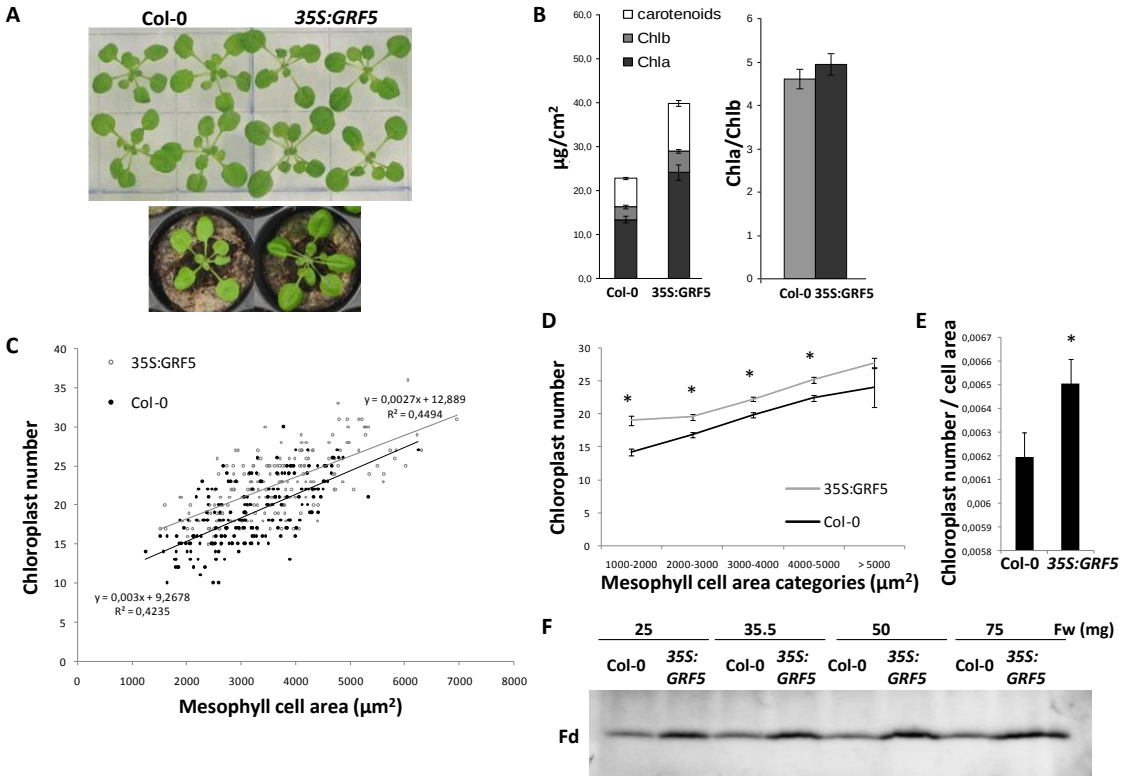


Figure 1. Overexpression of *GRF5* increases chloroplast number.

A, Rosettes of 21-day-old wild-type (Col-0) and *35S:GRF5* plants grown in vitro and in soil. B, Chlorophyll (Chl) and carotenoid content and Chla/Chlb ratio, measured in the fifth leaf of 21-day-old wild-type (Col-0) and *35S:GRF5* plants, grown in vitro under long day conditions (16h light/8h dark). Error bars indicate SD (n=4). C, Chloroplast number plotted in function of palisade mesophyll cell area. Therefore, micropscopic differential interference contrast (DIC) pictures were taken from peripendicular transverse sections of leaves 1&2 of wild-type and *35S:GRF5* plants grown for 21 days. The area of 200 mesophyll cells flanking the epidermis was measured with Image J from these sections, and the corresponding chloroplast number was determined. D, Average chloroplast number per palisade mesophyll cell area categorie. Chloroplast numbers were determined as described in C. E, Relative average chloroplast number per palisade mesophyll cell area. Chloroplast numbers were determined as described in C. Error bars indicate SE. * Significantly different from WT ($P < 0.05$, Student's *t* test). F, Ferredoxin (Fd) levels determined by SDS-PAGE and immunoblotting of chloroplast protein extracts, with anti-Fd antiserum. The gel was loaded by equal leaf fresh weight (Fw).

The increased chloroplast number is also reflected by enhanced levels of the plastid marker ferredoxin (Fd) relative to leaf fresh weight, in *35S:GRF5* compared to Col-0 chloroplast preparations, as determined by Western blot (Fig. 1, F). Fd is a multifunctional electron carrier that delivers reducing equivalents from PSI for the photoreduction of $NADP^+$, generating NADPH needed for CO_2 fixation and other biosynthetic processes (Knaff, 2004). However, no changes in Fd levels could be detected when the same amount of soluble proteins from total protein extracts were loaded, neither when equal chlorophyll loading from chloroplast extracts was performed (Supplemental Fig. 1). These data indicate that although *GRF5* overexpression increases the number of chloroplasts per cell, their structure may be unaffected.

Consistent with the latter observation, the ratio Chla/Chlb, reflecting a general overview of the photosynthetic apparatus, failed to show differences in the photosystem II (PSII)/PSI ratio between WT and *35S:GRF5* (Fig. 1, B). Because Chla is a core component of the photosystems, while Chlb in addition seems to be required for protein accumulation in the photosystem-associated light harvesting complexes (LHCs) (Tanaka and Tanaka, 2011), unaltered Chla/Chlb ratios suggest the absence of modifications in the LHCs and hence in the PSII/PSI ratio. Since both photosystems are physically separated, with PSI mainly located in stromal lamellae and PSII in the closely stacked grana of the thylakoid (Dekker and Boekema, 2005), similar Chla/Chlb ratios also point towards unchanged conformations of the thylakoid membrane system in *35S:GRF5* plastids. Indeed, the ultrastructure of chloroplasts of *35S:GRF5* plants did not seem to differ from wild-type plants, as imaged by transmission electron microscopy (Fig. 2).

Taken together, *GRF5* overexpression increases the number of chloroplasts per cell, rather than promoting changes in the development of mesophyll tissue or chloroplast structure.

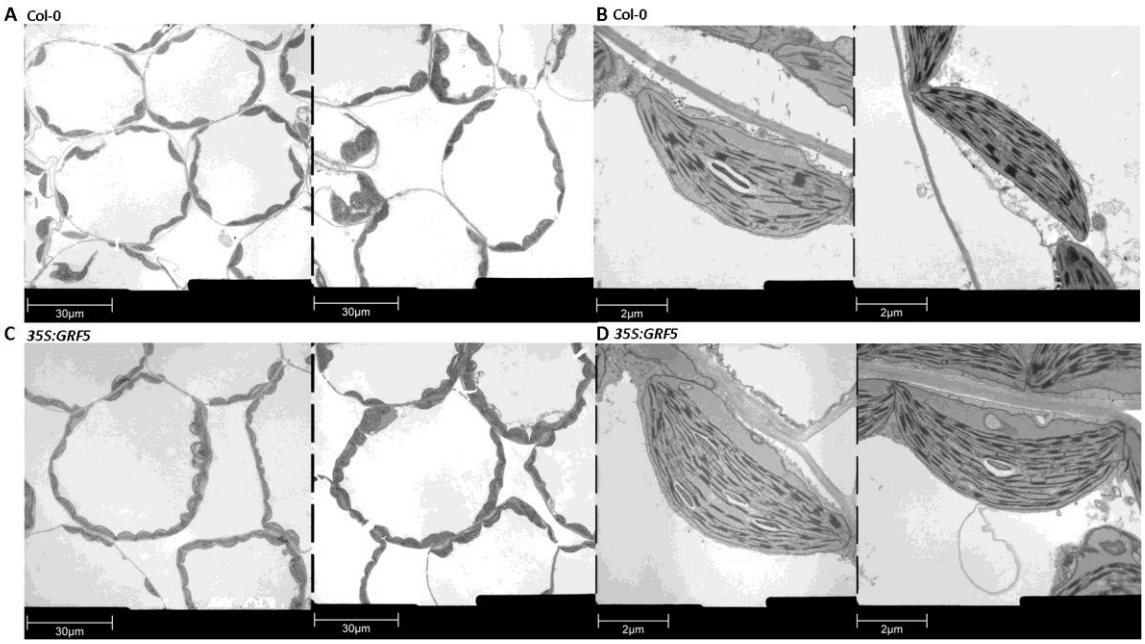


Figure 2. *35S:GRF5* mesophyll cells contain more chloroplast with similar ultrastructure.
Transmission electron micrographs of sections of 21 day-old leaves 1&2 of wild-type (Col-0) (A and B) and *35S:GRF5* (C and D) plants, grown in soil. Representative sections are shown at magnifications of 800x (A and C) or 10000x (B and D).

GRF5 influences photosynthetic capacity

To investigate if the increased amount of chloroplasts in the leaves of *GRF5* overexpressing plants can lead to an increase in photosynthesis, PSII fluorescence parameters were determined (Fig. 3, A to E). One month old wild-type and *35S:GRF5* leaves displayed similar maximum photochemical efficiency of PSII in the dark adapted state (Fv/Fm) and fluorescence quantum yield of PSII photochemistry (Y(II)), although *35S:GRF5* plants showed significantly increased electron transport rate (ETR) at higher light intensities (Fig. 3, A to C). Also a significant increase in photochemical quenching could be observed, while non-photochemical quenching (qN) was the same as in wild-type leaves (Fig. 3, D and E).

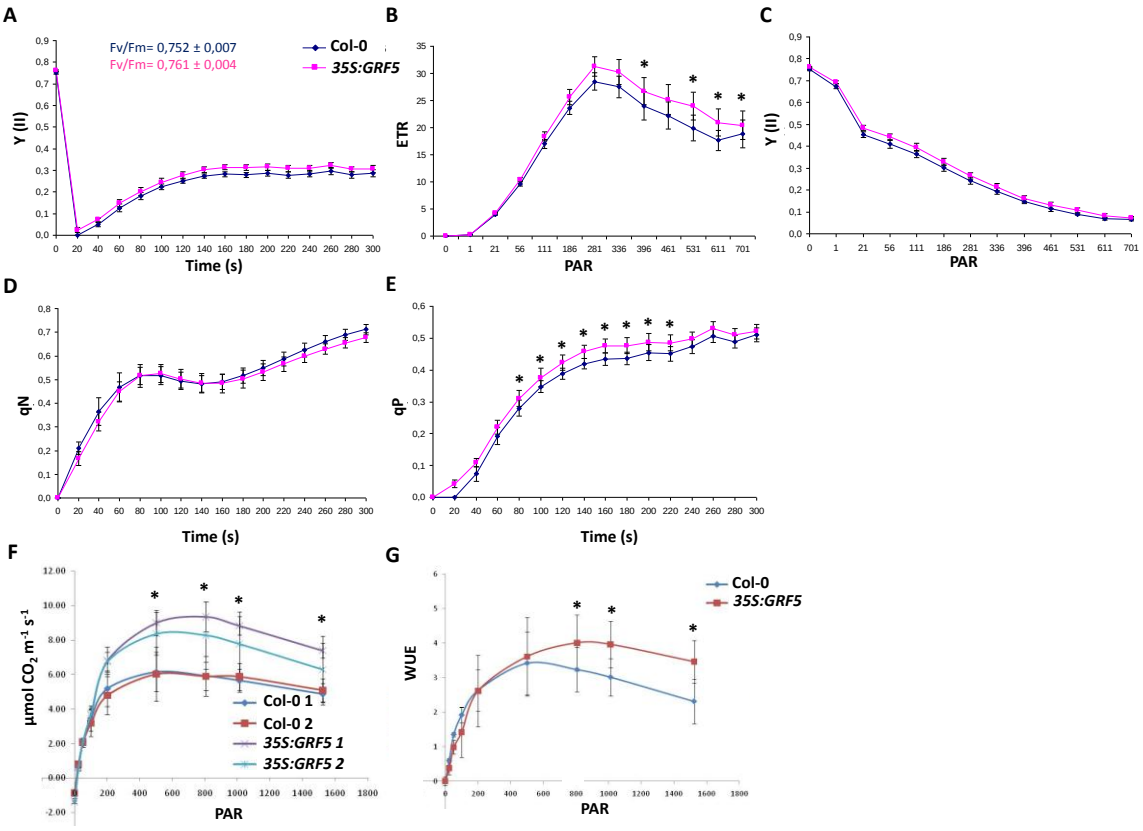


Figure 3. Altered photosynthetic capacity in *35S:GRF5* plants.

A to E, Photosynthetic parameters were determined by measurements of Chla fluorescence in wild-type and *35S:GRF5* leaves grown for one month in long day conditions in vitro. A, Y(II): quantum yield of photosystem II (PSII) photochemistry in function of the time. Fv/Fm: maximum photochemical efficiency of PSII in the dark adapted state. B, ETR: electron transport rate through PSII. PAR: photoactive radiation (µmol photons m^{-2} s^{-1}). C, Y(II): quantum yield of PSII in function of PAR. D, qN: non-photochemical quenching. E, qP: photochemical quenching. Data are means ± SD from 3 independent experiments (n = 10). F and G, Photosynthetic activity was determined from 21-day-old plants grown in short day conditions (8h light/16h dark). F, CO_2 assimilation measured for two different leaves from wild type (WT) and *35S:GRF5* (GRF5). G, WUE: water use efficiency calculated from max CO_2 assimilation (µmol CO_2 m^{-2} s^{-1}) divided by the transpiration (µmol H_2O m^{-2} s^{-1}). Error bars in F and G are SD (n = 3). * Significantly different from the wild type ($P \leq 0.005$, Student's *t* test).

Furthermore, *35S:GRF5* plants can sustain a higher maximum rate of CO_2 assimilation compared to the wild type, from light intensities higher than 200 µmol photons m^{-2} s^{-1} onwards (10.60 ± 0.14 vs 7.34 ± 0.64 µmol CO_2 m^{-2} s^{-1}), as measured in two different leaves for both wild-type and *35S:GRF5* plants (Fig. 3, F). However, the light saturation point (500 ± 100 µmol photons m^{-2} s^{-1}) and apparent quantum efficiency are similar in both lines (Fig. 3, F). Likewise, water use efficiency (WUE) is higher than in the wild type, but only at photoactive radiation > 560 µmol photons m^{-2} s^{-1} (Fig. 3, G), indicating that *35S:GRF5* leaves are able to assimilate higher amounts of CO_2 for a given amount of H_2O at high light intensities. Taken together, these data are in agreement with a similar structure and composition of the *35S:GRF5* and wild-type photosynthetic apparatus. Moreover, the increased number of chloroplasts per cell and increased Fd levels per fresh weight in *35S:GRF5* plants, are in line with the higher maximum rate of photosynthesis, determined by the rate of light-independent reactions, such as the calvin cycle and nitrogen assimilation pathways that require a steady supply of Fd for proper functioning (Knaff, 2004).

Cytokinins increase the ability of GRF5 to promote cell division

Because cytokinins are known for their positive role in chlorophyll synthesis and chloroplast development (Mok, 1994; Riefler et al., 2006; Argyros et al., 2008), the sensitivity of *35S:GRF5* plants to cytokinin treatment was investigated. Both GRF5 and cytokinins stimulate cell proliferation during leaf development (Werner et al., 2003; Horiguchi et al., 2005; Werner and Schmülling, 2009; Gonzalez et al., 2010; Holst et al., 2011). *35S:GRF5* was combined with the quantitative mitotic marker *CYCB1;1:D-Box-GUS-GFP* (*CYCB1;1:DB-GUS*) (Eloy et al., 2011), that allows for the identification of actively dividing cells (Colón-Carmona et al., 1999). Double homozygous *35S:GRF5*/*CYCB1;1:DB-GUS* and *CYCB1;1:DB-GUS* control plants were grown for 9 DAS and subsequently transferred to medium with different concentrations of the synthetic cytokinin 6-benzylaminopurine (BAP), for 24h, after which the first leaves were analyzed for GUS staining.

In the absence of BAP, mitotic activity was restricted to the basal part in *CYCB1;1:DB-GUS* leaves 1&2, whereas this GUS-stained region was extended along the length of the leaf in *35S:GRF5*/*CYCB1;1:DB-GUS* plants (Fig. 4, A and B). Although *GRF5* overexpression increased leaf length, the relative length of the division zone was significantly larger compared to control leaves (Fig. 4, A and B). In addition to measurement of the length of the GUS stained region, the intensity of the GUS staining was measured in a defined area along the leaf length, from the base to the tip of the leaf blade (Inset in Fig. 4, D; see materials and methods). This revealed that the GUS intensity was enhanced in *35S:GRF5*/*CYCB1;1:DB-GUS* leaves (Fig. 4, C, D and F), indicating that GRF5 most likely stimulates not only the duration but also the rate of cell proliferation during early leaf growth.

Exogenous application of 1µM BAP did not affect *CYCB1;1:DB-GUS* leaf length or GUS staining (Fig. 4, A, B and D, E), neither did it further extend the GUS-stained region in *35S:GRF5*/*CYCB1;1:DB-GUS* leaves, but it did increase the intensity of the GUS staining in the latter leaves after 24h (Fig. 4, A–C and E). A higher BAP concentration of 10µM promoted both the length and intensity of the GUS-stained region in *CYCB1;1:DB-GUS* and *35S:GRF5*/*CYCB1;1:DB-GUS* plants compared to untreated plants (Fig. 4, A-E). Whereas the increase in GUS intensity at 10µM BAP relative to the untreated plants, was as strong at the base of the leaves in both lines, it remained higher in a larger region along the length of the leaves in *35S:GRF5*/*CYCB1;1:DB-GUS* compared to *CYCB1;1:DB-GUS* plants (Fig. 4, E). Moreover, the relative increase in GUS intensity due to *GRF5* overexpression compared to control plants is synergistically enhanced by 10µM BAP treatment (Fig. 4, F).

Taken together, a BAP concentration as low as 1µM is only able to enhance the cell division rate when *GRF5* is overexpressed, while mitotic activity is not influenced in control plants. The higher BAP concentration of 10 µM stimulates the cell division rate and the duration of the cell proliferation phase in control and *35S:GRF5* plants, but the cell proliferation rate is enhanced in an extended region along the length of *GRF5* overexpressing leaves. Thus, ectopic expression of *GRF5* appears to increase the sensitivity to cytokinin-driven stimulation of cell proliferation, suggesting that GRF5 and cytokinins work together, as previously postulated (Vercruyssen et al., 2011).

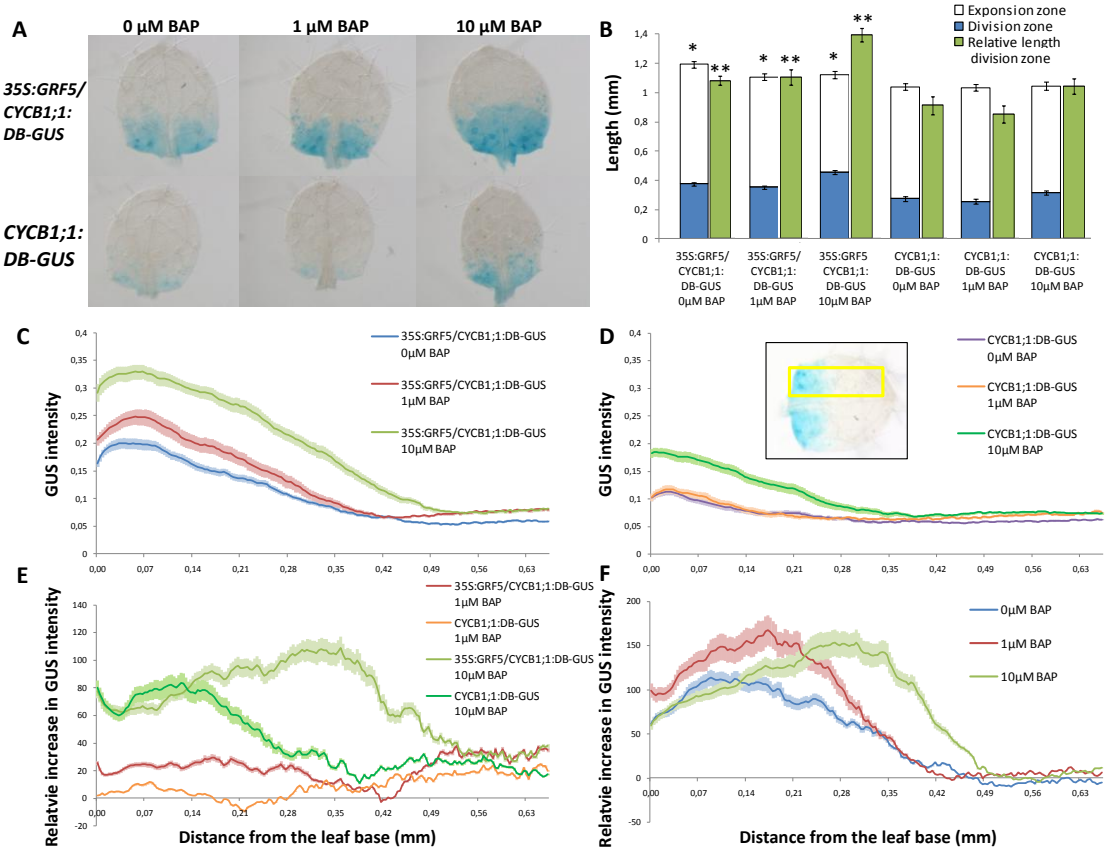


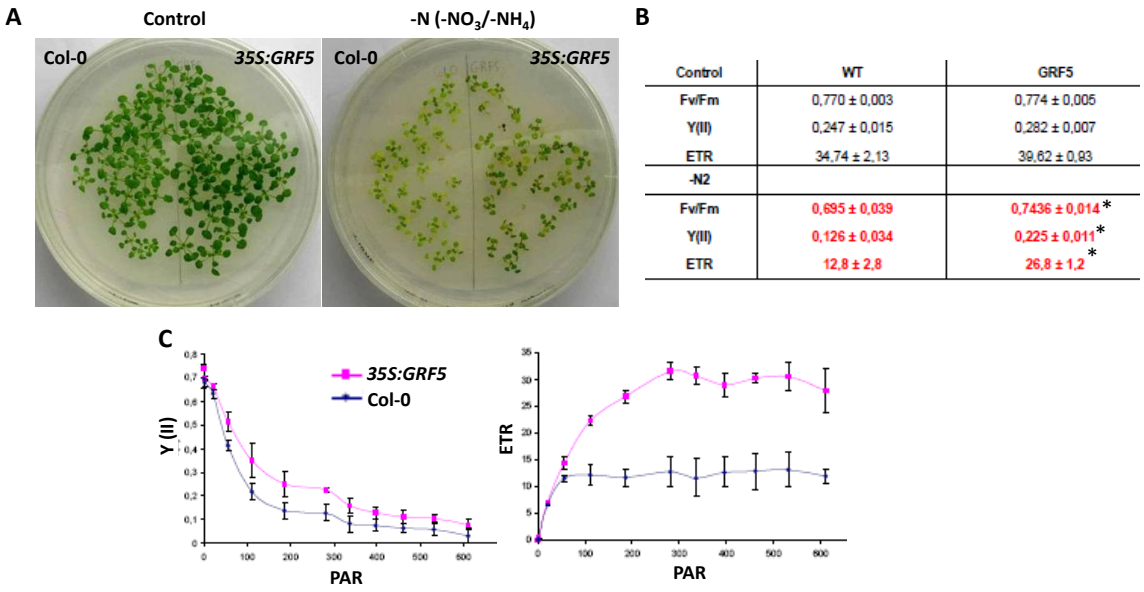
Figure 4. *35S:GRF5* plants show an increased region and intensity of *CYCB1;1* expression and are more susceptible to cytokinin treatment.

35S:GRF5/CYCB1;1:DB-GUS and *CYCB1;1:DB-GUS* control plants were grown on nylon meshes for 9 days in vitro, and subsequently transferred to medium with 0µM, 1µM and 10µM BAP for 24h hours before GUS staining. A, Leaves 1&2 were mounted on slides for picture taking. B, GUS-stained and non-stained regions, indicating the division and expansion zones, respectively, measured along the length of the leaf and the relative length of the GUS-stained zone in arbitrary units. * Significantly different from *CYCB1;1:DB-GUS* control plants at a similar BAP concentration. ** The relative length of the GUS-stainend zone is significantly different from *CYCB1;1:DB-GUS* control plants at a similar BAP concentration ($P < 0.05$, Student's *t* test). C-F, GUS staining was measured with Image J (see materials and methods) in a defined area along the leaf length, depicted by the yellow rectangle in the inset in D. C, GUS intensity in *35S:GRF5/CYCB1;1:DB-GUS* plants at 0µM, 1µM and 10µM BAP. D, GUS intensity in *CYCB1;1:DB-GUS* plants at 0µM, 1µM and 10µM BAP. E, Relative increase in GUS intensity by 1µM and 10µM BAP compared to 0µM BAP. F, Relative increase in GUS intensity of *35S:GRF5/CYCB1;1:DB-GUS* compared to *CYCB1;1:DB-GUS* at the different BAP concentrations. Error bars indicate SE ($n \geq 25$).

Worthwhile to mention, is that overexpression of *GRF5* lengthens the vegetative growth period by on average ten days under long day conditions (Supplemental Fig. 2). In addition, rosette leaves seem to stay darker green longer, suggesting that senescence is delayed by overexpression of *GRF5* (Supplemental Fig. 2). Both the transition to flowering and leaf senescence are also affected by cytokinins and could serve additional commonalities between putative GRF5 and cytokinin functions.

GRF5 overexpression increases tolerance for nitrogen deprivation

Cytokinins act as long-distance messengers signaling the nitrogen status of the plant and nitrogen assimilation occurs in part in the chloroplasts (Sakakibara et al., 2006; Lillo, 2008). The photosynthetic reduction of Fd is involved by distributing reducing power to nitrite reductase, that catalyses the conversion of nitrite to ammonium in the chloroplasts. Because of the increased chloroplast number and Fd levels per fresh weight in *35S:GRF5* leaves, the effect of nitrogen deficiency was studied. One week old *35S:GRF5* and wild-type seedlings were transferred to medium without $NH_4^+$and NO_3^-, lacking any source of nitrogen. As a control, the plants were simultaneously transferred to mock medium. After 12 days, *35S:GRF5* plants displayed increased tolerance against nitrogen deprivation as observed by the enhanced greening compared to control plants, concomitant with an increased photosynthetic capacity (Fig. 5, A to C). Values significantly higher than wild type for Fv/Fm, Y(II) and ETR could be measured in *35S:GRF5* plants after growth on medium without nitrogen (Fig. 5, B and C).



Control	WT	GRF5
Fv/Fm	0,770 ± 0,003	0,774 ± 0,005
Y(II)	0,247 ± 0,015	0,282 ± 0,007
ETR	34,74 ± 2,13	39,62 ± 0,93
-N2		
Fv/Fm	0,695 ± 0,039	0,7436 ± 0,014 *
Y(II)	0,126 ± 0,034	0,225 ± 0,011 *
ETR	12,8 ± 2,8	26,8 ± 1,2 *

Figure 5. *35S:GRF5* plants are more resistant to nitrogen deprivation.

A, Wild-type (Col-0) and *35S:GRF5* plants were grown for 7 days on normal ½ MS medium and subsequently transferred to control medium or medium completely depleted of nitrogen (-N; -NO_3/NH_4) for 12 days. B, Photosynthetic parameters at 300 µmol photons m^{-2} s^{-1} determined by measurements of Chla fluorescence of WT and *35S:GRF5* plants grown as described in A. Fv/Fm: maximum photochemical efficiency of PSII in the dark adapted state. Y(II): quantum yield of (PSII) photochemistry. ETR: electron transport rate through PSII. * Significantly different from the wild type ($P \leq 0.05$, Student's *t* test). C, Y(II) and ETR in function of photoactive radiation (PAR; µmol photons m^{-2} s^{-1}) in wild-type and *35S:GRF5* leaves after growth on N-deprived medium. Data are means ± SD of two independent experiments (n = 10).

DISCUSSION

GRF5 positively regulates leaf growth not only by stimulating cell division, but in addition by promoting chloroplast division. Overexpression of *GRF5* yields mesophyll cells that contain more chloroplasts, without affecting chloroplast structure. As a consequence, the leaves contain more chlorophyll and more Fd, resulting in an increased photosynthetic CO_2 uptake and enhanced ETR at higher light intensities. Furthermore, *35S:GRF5* plants develop tolerance to nitrogen deprivation. This could rely on increased nitrogen storage before transfer to nitrogen-depleted medium, due to more chloroplasts and higher Fd levels, directly involved in nitrogen assimilation. Alternatively, since

cytokinins are shown to work in concert with GRF5, the resistance to the lack of nitrogen could be caused by an enhanced cytokinin-dependent nitrogen uptake, mobilization or assimilation. Therefore, GRF5 could be a component of the complex cytokinin regulatory network involved in nitrogen metabolism.

In that respect, it is of particular interest to note that overexpression of *GNC* and *GNL*, both induced by cytokinin and nitrate, increase chlorophyll content and chloroplast number and could maintain higher chlorophyll levels relative to wild type under nitrogen deprivation, similarly to *35S:GRF5* plants (Hudson et al., 2011). GNC and GNL, are able to directly upregulate the expression of chloroplast-localized Fd-dependent *GLUTAMATE SYNTHASE 1* (*GLU1*), that converts ammonium into glutamate, thereby redirecting nitrogen assimilation towards increased chlorophyll production (Hudson et al., 2011). However, contrary to *GRF5* overexpression, *GNC* and *GNL* overexpression leads to reduced rosette growth (Hudson et al., 2011). Further studies are required to clarify a role, if any, for GNC and GNL during GRF5 function and its cross talk with cytokinin.

Nevertheless, cytokinins and GRF5 are both known to stimulate leaf cell proliferation. Several mechanisms can contribute to increase final leaf size and besides changes in cell expansion and meristemoid division, these include an increased cell division rate, a prolonged proliferation phase or more cells that are recruited from the SAM to form the leaf initials (Gonzalez et al., 2012). Previous analysis of *35S:GRF5* early leaf growth showed no differences in size of the first leaf primordia at 5 DAS, arguing against an increase in the number of cells that initiate the primordia (Ichihashi et al., 2010). Overexpression of *GRF5* increased the intensity as well as the region of *CYCB1;1:DB-GUS* marker gene expression, indicating that GRF5 acts to promote both the rate and the duration of cell proliferation. Cytokinins were also shown to delay the exit from the proliferation phase and to enhance the expression of mitotic *CYCD3* and *CYCB1;2* genes (Riou-Khamlichi et al., 1999; Dewitte et al., 2007; Holst et al., 2011; Steiner et al., 2012). In agreement, exogenous cytokinin application enhanced the intensity and region of *CYCB1;1:DB-GUS* expression, indicating that, similar to GRF5, cytokinins stimulate the cell division rate and the duration of the cell proliferation phase. Moreover, evidence is provided that cytokinins and GRF5 work together to stimulate these processes, since

cytokinin treatment and overexpression of *GRF5* synergistically increased *CYCB1;1:DB-GUS* levels. Remarkably, a recent publication describes similar synergistic increases for overexpression of class I *TEOSINTE BRANCHED1/CYCLOIDEA/PCF 14* (*TCP14*) and cytokinin treatment on *CYCB1;2:GUS* expression in inflorescences (Steiner et al., 2012). Moreover, enhanced cytokinin breakdown by ectopic expression of *CKX3* also suppresses several phenotypic effects of *TCP14* overexpression, similarly to what was observed for combined ectopic expression of *CKX3* and *GRF5* (Vercruyssen et al., 2011; Steiner et al., 2012). *TCP14* is expressed in young developing leaves (Kieffer et al., 2011) and therefore it will be very interesting to investigate a potential relationship between GRF5 and TCP14.

Given that GRF5 and cytokinins cooperate during leaf cell division, it is highly likely that they also work together to promote chloroplast division. The question remains what the putative molecular basis for this cross talk is. Comparison of transcript profiles from *35S:GRF5* seedlings with cytokinin treated seedlings and measurement of endogenous cytokinin concentrations in *35S:GRF5* seedlings, suggest that *GRF5* transcript levels are not directly influenced by cytokinin signaling nor that GRF5 affects cytokinin metabolism (Brenner et al., 2005; Nemhauser et al., 2006; Lee et al., 2007; Argyros et al., 2008; Gonzalez et al., 2010). The integration is rather accomplished through the regulation of common target genes. Of particular interest are *PORA* and *ALTERED MERISTEM PROGRAM 1* (*AMP1*), two genes that are differentially expressed in *35S:GRF5* seedlings (Gonzalez et al., 2010).

PORA is one of the three enzymes in Arabidopsis that catalyzes the light-dependent conversion of Pchlide to chlorophyllide, which is subsequently converted to chlorophyll, and its enzymatic activity requires NADPH generated by the Fd-dependent reduction of $NADP^+$ (Reinbothe et al., 1996; Oosawa et al., 2000; Su et al., 2001; Strasser et al., 2010). In the dark however, Pchlide accumulation requires the accumulation of PORs to bind Pchlide and prevent it from causing photooxidative damage upon light exposure in its unbound state (Sperling et al., 1997; op den Camp et al., 2003). PORA is strictly required for normal seedling growth, as photoautotrophy is never established in *porA* mutants, while *porB* and *porC* single mutants do not exhibit growth phenotypes (Frick et al., 2003; Masuda and Takamiya, 2004). Remarkably, *POR* mRNA levels were increased

strongly by cytokinin treatment in lupine (*Lupinus luteus L.*), concomitant with a faster greening of etiolated cotyledons (Kusnetsov et al., 1998) and also in cucumber (*Cucumis sativus, cv. aonagajibai*), *POR* was upregulated upon cytokinin treatment (Kuroda et al., 2000). This strongly suggests that also in Arabidopsis, *POR* levels could be controlled by cytokinins. *PORA* is the number one upregulated gene with the highest fold change in *35S:GRF5* seedlings, making it a putative point of convergence for GRF5 and cytokinin action in promoting chlorophyll synthesis (Gonzalez et al., 2010). Increased levels of the PORA substrate NADHP are to be expected from the higher Fd levels per fresh weight in *35S:GRF5* leaves, and probably contribute together with *PORA* upregulation to increased chlorophyll accumulation.

AMP1 encodes a putative glutamate carboxypeptidase with a thus far unknown substrate and *amp1* mutants have a higher endogenous cytokinin content (Chaudhury et al., 1993; Helliwell et al., 2001). As a consequence, they show faster leaf formation and an enlarged SAM (Chaudhury et al., 1993; Saibo et al., 2007). However, other phenotypes are observed that cannot be attributed to increased cytokinin levels, but that are potentially linked to other hormone signaling pathways like those of GA, ethylene or auxin (Vidaurre et al., 2007; Griffiths et al., 2011). Remarkably, *amp1* developing seeds have increased mRNA levels of *CRF2*, a target of MONOPTEROS/AUXIN RESPONSE FACTOR 5 (MP/ARF5), and consistently AMP1 counteracts MP during embryogenesis (Vidaurre et al., 2007; Griffiths et al., 2011). Since CRF2 was shown to promote chloroplast division, the increased chloroplast number of *35S:GRF5* plants could be caused by differential expression of *AMP1* (Okazaki et al., 2009). However, *AMP1* is induced in *35S:GRF5* and repressed in *35S:CKX1* seedlings, in contrast to what would be expected (Brenner et al., 2005; Gonzalez et al., 2010). Therefore, the potential common regulation of *AMP1* by GRF5 and cytokinins needs further investigation.

The delayed flowering of *GRF5* overexpressing plants is in agreement with previous reports on other *GRFs* (Kim et al., 2003) and coincides putatively with a delay in the onset of senescence. Although cytokinins play a positive role in delaying senescence, their requirement for flowering is less clear, but recently shown to be rather stimulating than inhibiting (D'Aloia et al., 2011). Remarkably, *amp1* mutants display early flowering, which could be consistent with the late flowering phenotype and upregulation of the gene

in *35S:GRF5* plants (Chaudhury et al., 1993). Future research on *AMP1* and the previously mentioned genes as potential downstream GRF5 targets should shed light on the molecular basis of the *35S:GRF5* phenotypes.

The molecular mechanisms by which GRF5 stimulates cell division and chloroplast division remain to be uncovered. The cell cycle and plastid division were shown to be linked during leaf development and likewise, GRF5 could promote cell and chloroplast division by the same molecular mechanism resulting in the coordination of both processes (Raynaud et al., 2005). In addition, it is tempting to speculate that the enhanced potential for carbon and nitrogen assimilation contributes to the growth increase in *35S:GRF5* plants. Furthermore, enhanced nitrogen use efficiency has become an important biotechnological trait for the genetic improvement of crops, especially due to the detrimental effects on the environment and high cost of nitrogen fertilizers (Edgerton, 2009; Kant et al., 2011). By cross talk with cytokinins, *GRF5* could help integrating external signals like light and nutrients with the endogenous requirements for growth and be a valuable candidate for genetic engineering into crop species.

MATERIALS AND METHODS

Plant material and growth conditions

35S:GRF5 seeds were kindly provided by Prof. Dr. Hirokazu Tsukaya (Horiguchi et al., 2005). Double homozygous *35S:GRF5/CYCB1;1:DB-GUS* plants were obtained by crossing of *35S:GRF5* plants with *CYCB1;1:DB-GUS* plants (Eloy et al., 2011) followed by selfing and selection based on hygromycin and kanamycin resistance, respectively. Both lines are in the *Arabidopsis thaliana* (L.) Heyhn. ecotype Columbia (Col-0) background.

For *in vitro* experiments, seeds were sown in sterile plates containing half-strength Murashige and Skoog (½MS) medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.8% agar. The plates were sealed and put in a tissue culture room at 21°C under a 16h day/8h night regime. For experiments in soil, the plants were grown at 22°C under long day (16h day/8h night) or short day (8h day/16h night) conditions (50 μmol m^{-2} s^{-1}).

Chloroplast analysis

Soil-grown plants were harvested at 21 DAS and peripendicular transverse sections were made of leaves 1&2 and mounted on slides, according to a protocol described previously (Skirycz et al., 2010). Micropscopic differential interference contrast (DIC) pictures were taken and the area of 200 mesophyll cells flanking the epidermis was measured with the ImageJ software (http://rsb.info.nih.gov/ij/), and the corresponding chloroplast number was determined. For transmission electron microscopy, ultrathin sections were prepared as described previously (Skirycz et al., 2010).

Intact chloroplasts were isolated from wild-type and transgenic plants using Percoll gradient centrifugation (Wu et al., 1991). Chlorophyll in leaves and chloroplasts was determined spectrophotometrically in acetone extracts (Lichtenthaler, 1987). Detection of Fd in chloroplast and leaf extracts was performed by SDS-PAGE and immunoblotting using 15% polyacrylamide gels and polyclonal antiserum raised in rabbits.

Photosynthetic activity determinations

Chla fluorescence measurements were performed at 25°C on *in vitro* grown dark-adapted seedlings using an Imaging-PAM M-Series chlorophyll fluorescence System (Heinz Walz). The Fv and Fm parameters were determined after 30 min in the dark. Photosynthetic parameters (Fv/Fm, Y(II), ETR and non-photochemical and photochemical quenching) were calculated as described (Baker and Rosenqvist, 2004; Baker, 2008).

Light-dependent CO_2 assimilation (Amax, μmol CO_2 m^{-2} s^{-1}) and transpiration (E, μmol H_2O m^{-2} s^{-1}) were determined on fully expanded attached leaves of 3-week-old plants grown in soil under short day growth conditions (2 leaves of 3 plants of each line) using an GFS-3000 portable photosynthesis system from (Heinz Walz). The CO_2 concentration of the air entering the leaf chamber and the temperature were adjusted to 360 ppm and 25°C, respectively. PPFD ranging from 50 to 1500 mmol m^{-2} s^{-1} was supplied by a controlled halogen light source. The water use efficiency parameters (WUE, μmol CO_2/mol H_2O) were calculated from A/E. The data was further analyzed by Photosyn Assistant (http://www.scientific.force9.co.uk/photosyn.htm)

GUS staining and analysis

For cytokinin treatments, the plates containing control medium were overlaid with nylon meshes (Prosep) of 20-μm pore size to prevent roots from growing into the medium, after which seeds were sown. At 9 DAS, seedlings were transferred by gently lifting the nylon mesh with forceps to plates containing mock medium or medium supplemented with different concentrations of 6-benzylaminopurine (BAP).

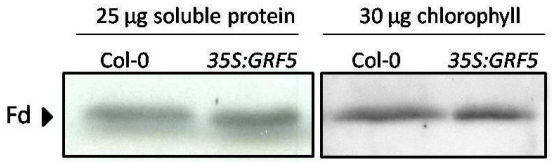
Seedlings were harvested at 10 DAS 24h after BAP treatment and incubated in heptane for 10 min and subsequently left to dry for 5 min. Then they were submersed in 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc) buffer [100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl, 50mM NaCl buffer (pH 7.0), 2mM $K_3[Fe(CN)_6]$, and 4mM X-Gluc], vacuum infiltrated for 10 min and incubated at 37°C for 8 h. Seedlings were cleared in 100% and 70% ethanol and then kept in 90% lactic acid. Leaves 1&2 were mounted on slides and photographed under a stereomicroscope.

Leaf length and GUS staining was measured with the ImageJ software (http://rsb.info.nih.gov/ij/). The leaves were imaged in a horizontal position, the background was subtracted and a defined area along the length of the leaf was selected with the rectangle tool. Next, the color intensity in the rectangle was measured with a one-pixel resolution using the plot profile function. The data points were then calibrated by adjusting the distance from pixels to millimeters, and the color intensities were normalized to an arbitrary scale of 0 to 1.

Nitrogen depletion assays

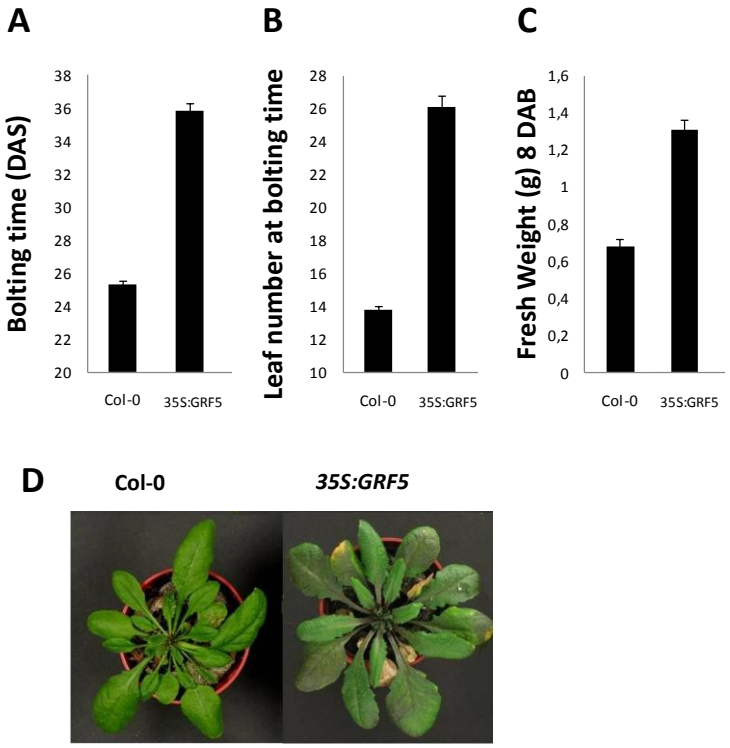
For *in vitro* survival assays under nitrogen-free growth conditions, 7-day-old seedlings grown on control medium were transferred for 12 days to plates without nitrogen. Thereto, ammonium nitrate and potassium nitrate were replaced by 18.79 mM potassium chloride.

SUPPLEMENTAL DATA



Supplemental figure 1. Ferredoxin levels in total leaf protein and chloroplast extracts.

Ferredoxin (Fd) levels determined by SDS-PAGE and immunoblotting with anti-Fd antiserum of total soluble protein extracts (left) and chloroplast protein extracts (right) of wild-type (Col-0) and *35S:GRF5* plants. The gel was loaded by equal amount of soluble protein (left), corresponsing to 2.3mg leaf fresh weight for Col-0 and 1.1mg for *35S:GRF5*, or by equal amount of chlorophyll (right).



Supplemental figure 2. *GRF5* ectopic expression delays flowering.

A, Bolting time, expressed as days after stratification (DAS) of wild-type and *GRF5* overexpressing plants, grown in soil in 16h light/8h dark conditions. B, Leaf number at the time of bolting. C, Rosette fresh weight, after removal of the inflorescence stem on average 8 days after bolting (8 DAB). This corresponds to 35 DAS for Col-0, and to 42 DAS for *35S:GRF5* plants. D, Rosettes, on average 8 DAB, correspoding to DAS as described in C. The inflorescence stem was removed before picture taking. Error bars indicate SE ($n \geq 24$ [A and B] and $n \geq 13$ [C]).

REFERENCES

Andriankaja M, Dhondt S, De Bodt S, Vanhaeren H, Coppens F, De Milde L, Mühlenbock P, Skirycz A, Gonzalez N, Beemster GTS, Inzé D (2012) Exit from proliferation during leaf development in *Arabidopsis thaliana*: a not-so-gradual process. Developmental Cell **22:** 64-78

Argueso CT, Ferreira FJ, Kieber JJ (2009) Environmental perception avenues: the interaction of cytokinin and environmental response pathways. Plant Cell and Environment **32:** 1147-1160

Argyros RD, Mathews DE, Chiang YH, Palmer CM, Thibault DM, Etheridge N, Argyros DA, Mason MG, Kieber JJ, Schaller GE (2008) Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. Plant Cell **20:** 2102-2116

Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annual Review of Plant Biology **59:** 89-113

Baker NR, Rosenqvist E (2004) Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. J Exp Bot **55:** 1607-1621

Beemster GTS, De Veylder L, Vercruysse S, West G, Rombaut D, Van Hummelen P, Galichet A, Gruissem W, Inzé D, Vuylsteke M (2005) Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. Plant Physiology **138:** 734-743

Bi YM, Zhang Y, Signorelli T, Zhao R, Zhu T, Rothstein S (2005) Genetic analysis of Arabidopsis GATA transcription factor gene family reveals a nitrate-inducible member important for chlorophyll synthesis and glucose sensitivity. Plant Journal **44:** 680-692

Boonman A, Prinsen E, Gilmer F, Schurr U, Peeters AJM, Voesenek LACJ, Pons TL (2007) Cytokinin import rate as a signal for photosynthetic acclimation to canopy light gradients. Plant Physiol **143:** 1841-1852

Brenner WG, Ramireddy E, Heyl A, Schmulling T (2012) Gene regulation by cytokinin in Arabidopsis. Front Plant Sci **3:** 8

Brenner WG, Romanov GA, Kollmer I, Burkle L, Schmulling T (2005) Immediate-early and delayed cytokinin response genes of Arabidopsis thaliana identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant Journal **44:** 314-333

Chaudhury AM, Letham S, Craig S, Dennis ES (1993) Amp1 - a Mutant with High Cytokinin Levels and Altered Embryonic Pattern, Faster Vegetative Growth, Constitutive Photomorphogenesis and Precocious Flowering. Plant Journal **4:** 907-916

Chory J, Reinecke D, Sim S, Washburn T, Brenner M (1994) A role for cytokinins in de-etiolation in *Arabidopsis*: *det* mutants have an altered response to cytokinins. Plant Physiology **104:** 339-347

Colón-Carmona A, You R, Haimovitch-Gal T, Doerner P (1999) Spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. Plant Journal **20:** 503-508

Cutcliffe JW, Hellmann E, Heyl A, Rashotte AM (2011) CRFs form protein-protein interactions with each other and with members of the cytokinin signalling pathway in Arabidopsis via the CRF domain. J Exp Bot **62:** 4995-5002

D'Aloia M, Bonhomme D, Bouche F, Tamseddak K, Ormenese S, Torti S, Coupland G, Perilleux C (2011) Cytokinin promotes flowering of Arabidopsis via transcriptional activation of the FT paralogue TSF. Plant Journal **65:** 972-979

Dekker JP, Boekema EJ (2005) Supramolecular organization of thylakoid membrane proteins in green plants. Biochimica Et Biophysica Acta-Bioenergetics **1706:** 12-39

Dewitte W, Scofield S, Alcasabas AA, Maughan SC, Menges M, Braun N, Collins C, Nieuwland J, Prinsen E, Sundaresan V, Murray JAH (2007) *Arabidopsis* CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proceedings of the National Academy of Sciences of the United States of America **104:** 14537-14542

Donnelly PM, Bonetta D, Tsukaya H, Dengler RE, Dengler NG (1999) Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. Developmental Biology **215:** 407-419

Dortay H, Gruhn N, Pfeifer A, Schwerdtner M, Schmulling T, Heyl A (2008) Toward an interaction map of the two-component signaling pathway of Arabidopsis thaliana. Journal of Proteome Research **7:** 3649-3660

Dortay H, Mehnert N, Burkle L, Schmulling T, Heyl A (2006) Analysis of protein interactions within the cytokinin-signaling pathway of Arabidopsis thaliana. Febs Journal **273:** 4631-4644

Edgerton MD (2009) Increasing Crop Productivity to Meet Global Needs for Feed, Food, and Fuel. Plant Physiol **149:** 7-13

Eloy NB, de Freitas Lima M, Van Damme D, Vanhaeren H, Gonzalez N, De Milde L, Hemerly AS, Beemster GTS, Inzé D, Ferreira PCG (2011) The APC/C *subunit 10* plays an essential role in cell proliferation during leaf development. Plant Journal **68:** 351-363

Frick G, Su QX, Apel K, Armstrong GA (2003) An Arabidopsis porB porC double mutant lacking light-dependent NADPH : protochlorophyllide oxidoreductases B and C is highly chlorophyll-deficient and developmentally arrested. Plant Journal **35:** 141-153

Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhondt S, Van Daele T, De Milde L, Weigel D, Kamiya Y, Stitt M, Beemster GTS, Inzé D (2010) Increased leaf size: different means to an end. Plant Physiology **153:** 1261-1279

Gonzalez N, Vanhaeren H, Inzé D (2012) Leaf size control: complex coordination of cell division and expansion. Trends in Plant Science **in press**

Griffiths J, Barrero JM, Taylor J, Helliwell CA, Gubler F (2011) ALTERED MERISTEM PROGRAM 1 Is involved in Development of Seed Dormancy in Arabidopsis. Plos One **6**

Hedtke B, Alawady A, Albacete A, Kobayashi K, Melzer M, Roitsch T, Masuda T, Grimm B (2012) Deficiency in riboflavin biosynthesis affects tetrapyrrole biosynthesis in etiolated Arabidopsis tissue. Plant Mol Biol **78:** 77-93

Helliwell CA, Chin-Atkins AN, Wilson IW, Chapple R, Dennis ES, Chaudhury A (2001) The Arabidopsis AMP1 gene encodes a putative glutamate carboxypeptidase. Plant Cell **13:** 2115-2125

Holst K, Schmulling T, Werner T (2011) Enhanced cytokinin degradation in leaf primordia of transgenic Arabidopsis plants reduces leaf size and shoot organ primordia formation. Journal of Plant Physiology **168:** 1328-1334

Horiguchi G, Kim G-T, Tsukaya H (2005) The transcription factor AtGRF5 and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of *Arabidopsis thaliana*. Plant Journal **43:** 68-78

Hudson D, Guevara D, Yaish MW, Hannam C, Long N, Clarke JD, Bi YM, Rothstein SJ (2011) GNC and CGA1 Modulate Chlorophyll Biosynthesis and Glutamate Synthase (GLU1/Fd-GOGAT) Expression in Arabidopsis. Plos One **6**

Hutchison CE, Li J, Argueso C, Gonzalez M, Lee E, Lewis MW, Maxwell BB, Perdue TD, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2006) The Arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. Plant Cell **18:** 3073-3087

Hwang I, Sheen J (2001) Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature **413:** 383-389

Hwang I, Sheen J, Muller B (2012) Cytokinin signaling networks. Annu Rev Plant Biol **63:** 353-380

Ichihashi Y, Horiguchi G, Gleissberg S, Tsukaya H (2010) The bHLH Transcription Factor SPATULA Controls Final Leaf Size in Arabidopsis thaliana. Plant and Cell Physiology **51:** 252-261

Ishida K, Yamashino T, Yokoyama A, Mizuno T (2008) Three type-B response regulators, ARR1, ARR10 and ARR12, play essential but redundant roles in cytokinin signal transduction throughout the life cycle of Arabidopsis thaliana. Plant and Cell Physiology **49:** 47-57

Kakimoto T (2001) Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate : ATP/ADP isopentenyltransferases. Plant and Cell Physiology **42:** 677-685

Kant S, Bi YM, Rothstein SJ (2011) Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. J Exp Bot **62:** 1499-1509

Kazama T, Ichihashi Y, Murata S, Tsukaya H (2010) The mechanism of cell cycle arrest front progression explained by a *KLUH/CYP78A5*-dependent mobile growth factor in developing leaves of *Arabidopsis thaliana*. Plant and Cell Physiology **51:** 1046-1054

Kiba T, Kudo T, Kojima M, Sakakibara H (2011) Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. J Exp Bot **62:** 1399-1409

Kiba T, Naitou T, Koizumi N, Yamashino T, Sakakibara H, Mizuno T (2005) Combinatorial microarray analysis revealing Arabidopsis genes implicated in cytokinin responses through the His -> Asp phosphorelay circuitry. Plant and Cell Physiology **46:** 339-355

Kiba T, Yamada H, Sato S, Kato T, Tabata S, Yamashino T, Mizuno T (2003) The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in Arabidopsis thaliana. Plant and Cell Physiology **44:** 868-874

Kieffer M, Master V, Waites R, Davies B (2011) TCP14 and TCP15 affect internode length and leaf shape in Arabidopsis. Plant Journal **68:** 147-158

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **103:** 814-819

Kim JH, Choi D, Kende H (2003) The AtGRF family of putative transcription factors is involved in leaf and cotyledon growth in *Arabidopsis*. Plant Journal **36:** 94-104

Kim JH, Lee BH (2006) GROWTH-REGULATING FACTOR4 of Arabidopsis thaliana is required for development of leaves, cotyledons, and shoot apical meristem. Journal of Plant Biology **49:** 463-468

Kim K, Ryu H, Cho YH, Scacchi E, Sabatini S, Hwang I (2012) Cytokinin-facilitated proteolysis of ARABIDOPSIS RESPONSE REGULATOR 2 attenuates signaling output in two-component circuitry. Plant Journal **69:** 934-945

Knaff DB (2004) Ferrdoxin and ferredoxin-dependent enzymes. *In* DR Ort, CF Yocum, IF Heichel, eds, Oxygenic Photosynthesis: The Light Reactions, Vol 4. Springer Netherlands, Dordrecht, The Netherlands, pp 333–361

Kollmer I, Werner T, Schmulling T (2011) Ectopic expression of different cytokinin-regulated transcription factor genes of Arabidopsis thaliana alters plant growth and development. Journal of Plant Physiology **168:** 1320-1327

Kudo T, Kiba T, Sakakibara H (2010) Metabolism and Long-distance Translocation of Cytokinins. Journal of Integrative Plant Biology **52:** 53-60

Kuroda H, Masuda T, Fusada N, Ohta H, Takamiya K (2000) Expression of NADPH-protochlorophyllide oxidoreductase gene in fully green leaves of cucumber. Plant and Cell Physiology **41:** 226-229

Kuroha T, Tokunaga H, Kojima M, Ueda N, Ishida T, Nagawa S, Fukuda H, Sugimoto K, Sakakibara H (2009) Functional Analyses of LONELY GUY Cytokinin-Activating Enzymes Reveal the Importance of the Direct Activation Pathway in Arabidopsis. Plant Cell **21:** 3152-3169

Kusnetsov V, Herrmann RG, Kulaeva ON, Oelmuller R (1998) Cytokinin stimulates and abscisic acid inhibits greening of etiolated Lupinus luteus cotyledons by affecting the expression of the light-sensitive protochlorophyllide oxidoreductase. Molecular and General Genetics **259:** 21-28

Lee DJ, Kim S, Ha YM, Kim J (2008) Phosphorylation of Arabidopsis response regulator 7 (ARR7) at the putative phospho-accepting site is required for ARR7 to act as a negative regulator of cytokinin signaling. Planta **227:** 577-587

Lee DJ, Park JY, Ku SJ, Ha YM, Kim S, Kim MD, Oh MH, Kim J (2007) Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7(ARR7) overexpression in cytokinin response. Molecular Genetics and Genomics **277:** 115-137

Lichtenthaler HK (1987) Chlorophylls and Carotenoids - Pigments of Photosynthetic Biomembranes. Methods in Enzymology **148:** 350-382

Lillo C (2008) Signalling cascades integrating light-enhanced nitrate metabolism. Biochemical Journal **415:** 11-19

Mason MG, Mathews DE, Argyros DA, Maxwell BB, Kieber JJ, Alonso JM, Ecker JR, Schaller GE (2005) Multiple type-B response regulators mediate cytokinin signal transduction in *Arabidopsis*. Plant Cell **17:** 3007-3018

Masuda T, Takamiya K (2004) Novel insights into the enzymology, regulation and physiological functions of light-dependent protochlorophyllide oxidoreductase in angiosperms. Photosynthesis Research **81:** 1-29

Miller CO, Skoog F, Vonsaltza MH, Strong FM (1955) Kinetin, a Cell Division Factor from Deoxyribonucleic Acid. Journal of the American Chemical Society **77:** 1392-1392

Miyawaki K, Matsumoto-Kitano M, Kakimoto T (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant Journal **37:** 128-138

Miyawaki K, Tarkowski P, Matsumoto-Kitano M, Kato T, Sato S, Tarkowska D, Tabata S, Sandberg G, Kakimoto T (2006) Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA

isopentenyltransferases in cytokinin biosynthesis. Proceedings of the National Academy of Sciences of the United States of America **103:** 16598-16603

Mok M (1994) Cytokinins and plant development: an overview. *In* D Mok, M Mok, eds, Cytokinins: chemistry, activity, and function, Boca Raton, FL: CRC, pp 155-166

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum **15:** 473-497

Naito T, Kiba T, Koizumi N, Yamashino T, Mizuno T (2007) Characterization of a unique GATA family gene that responds to both light and cytokinin in Arabidopsis thaliana. Bioscience Biotechnology and Biochemistry **71:** 1557-1560

Nemhauser JL, Hong F, Chory J (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell **126:** 467-475

Okazaki K, Kabeya Y, Suzuki K, Mori T, Ichikawa T, Matsui M, Nakanishi H, Miyagishima S (2009) The PLASTID DIVISION1 and 2 Components of the Chloroplast Division Machinery Determine the Rate of Chloroplast Division in Land Plant Cell Differentiation. Plant Cell **21:** 1769-1780

Oosawa N, Masuda T, Awai K, Fusada N, Shimada H, Ohta H, Takamiya K (2000) Identification and light-induced expression of a novel gene of NADPH-protochlorophyllide oxidoreductase isoform in Arabidopsis thaliana. Febs Letters **474:** 133-136

op den Camp RGL, Przybyla D, Ochsenbein C, Laloi C, Kim CH, Danon A, Wagner D, Hideg E, Gobel C, Feussner I, Nater M, Apel K (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in arabidopsis. Plant Cell **15:** 2320-2332

Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2006) A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proceedings of the National Academy of Sciences of the United States of America **103:** 11081-11085

Raynaud C, Perennes C, Reuzeau C, Catrice O, Brown S, Bergounioux C (2005) Cell and plastid division are coordinated through the prereplication factor AtCDT1. Proceedings of the National Academy of Sciences of the United States of America **102:** 8216-8221

Reinbothe S, Reinbothe C, Lebedev N, Apel K (1996) PORA and PORB, two light-dependent protochlorophyllide-reducing enzymes of angiosperm chlorophyll biosynthesis. Plant Cell **8:** 763-769

Ren B, Liang Y, Deng Y, Chen QG, Zhang J, Yang XH, Zuo JR (2009) Genome-wide comparative analysis of type-A Arabidopsis response regulator genes by overexpression studies reveals their diverse roles and regulatory mechanisms in cytokinin signaling. Cell Research **19:** 1178-1190

Richter R, Behringer C, Muller IK, Schwechheimer C (2010) The GATA-type transcription factors GNC and GNL/CGA1 repress gibberellin signaling downstream from DELLA proteins and PHYTOCHROME-INTERACTING FACTORS. Genes & Development **24:** 2093-2104

Riefler M, Novak O, Strnad M, Schmulling T (2006) Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18:** 40-54

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH (1999) Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science **283:** 1541-1544

Saibo NJM, Vriezen WH, De Grauwe L, Azmi A, Prinsen E, Van der Straeten D (2007) A comparative analysis of the Arabidopsis mutant amp1-1 and a novel weak amp1 allele reveals new functions of the AMP1 protein. Planta **225:** 831-842

Sakakibara H, Takei K, Hirose N (2006) Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends in Plant Science **11:** 440-448

Sakamoto W, Miyagishima SY, Jarvis P (2008) Chloroplast biogenesis: control of plastid development, protein import, division and inheritance. Arabidopsis Book **6:** e0110

Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D, Thimm O, Udvardi MK, Stitt M (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiol **136:** 2483-2499

Schoefs B, Franck F (2003) Protochlorophyllide reduction: Mechanisms and evolution. Photochemistry and Photobiology **78:** 543-557

Skirycz A, De Bodt S, Obata T, De Clercq I, Claeys H, De Rycke R, Andriankaja M, Van Aken O, Van Breusegem F, Fernie AR, Inzé D (2010) Developmental stage specificity and the role of

mitochondrial metabolism in the response of Arabidopsis leaves to prolonged mild osmotic stress. Plant Physiology **152:** 226-244

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symposia of the Society for Experimental Biology **11:** 118-130

Sperling U, vanCleve B, Frick G, Apel K, Armstrong GA (1997) Overexpression of light-dependent PORA or PORB in plants depleted of endogenous POR by far-red light enhances seedling survival in white light and protects against photooxidative damage. Plant Journal **12:** 649-658

Steiner E, Efroni I, Gopalraj M, Saathoff K, Tseng TS, Kieffer M, Eshed Y, Olszewski N, Weiss D (2012) The Arabidopsis O-Linked N-Acetylglucosamine Transferase SPINDLY Interacts with Class I TCPs to Facilitate Cytokinin Responses in Leaves and Flowers. Plant Cell **24:** 96-108

Strasser B, Sanchez-Lamas M, Yanovsky MJ, Casal JJ, Cerdan PD (2010) Arabidopsis thaliana life without phytochromes. Proceedings of the National Academy of Sciences of the United States of America **107:** 4776-4781

Su QX, Frick G, Armstrong G, Apel K (2001) POR C of Arabidopsis thaliana: a third light- and NADPH-dependent protochlorophyllide oxidoreductase that is differentially regulated by light. Plant Mol Biol **47:** 805-813

Takei K, Sakakibara H, Sugiyama T (2001) Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in Arabidopsis thaliana. Journal of Biological Chemistry **276:** 26405-26410

Takei K, Ueda N, Aoki K, Kuromori T, Hirayama T, Shinozaki K, Yamaya T, Sakakibara H (2004a) AtIPT3 is a key determinant of nitrate-dependent cytokinin biosynthesis in Arabidopsis. Plant and Cell Physiology **45:** 1053-1062

Takei K, Yamaya T, Sakakibara H (2004b) Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-Zeatin. Journal of Biological Chemistry **279:** 41866-41872

Tanaka R, Tanaka A (2011) Chlorophyll cycle regulates the construction and destruction of the light-harvesting complexes. Biochimica Et Biophysica Acta-Bioenergetics **1807:** 968-976

Taniguchi M, Sasaki N, Tsuge T, Aoyama T, Oka A (2007) ARR1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions. Plant and Cell Physiology **48:** 263-277

To JPC, Haberer G, Ferreira FJ, Deruere J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2004) Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. Plant Cell **16:** 658-671

Tokunaga H, Kojima M, Kuroha T, Ishida T, Sugimoto K, Kiba T, Sakakibara H (2012) Arabidopsis lonely guy (LOG) multiple mutants reveal a central role of the LOG-dependent pathway in cytokinin activation. Plant Journal **69:** 355-365

Vandenbussche F, Habricot Y, Condiff AS, Maldiney R, Van der Straeten D, Ahmad M (2007) HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in *Arabidopsis thaliana*. Plant Journal **49:** 428-441

Vercruyssen L, Gonzalez N, Werner T, Schmulling T, Inze D (2011) Combining Enhanced Root and Shoot Growth Reveals Cross Talk between Pathways That Control Plant Organ Size in Arabidopsis. Plant Physiol **155:** 1339-1352

Vidaurre DP, Ploense S, Krogan NT, Berleth T (2007) AMP1 and MP antagonistically regulate embryo and meristem development in Arabidopsis. Development **134:** 2561-2567

Wareing PF, Khalifa MM, Treharne KJ (1968) Rate-Limiting Processes in Photosynthesis at Saturating Light Intensities. Nature **220:** 453-&

Werner T, Holst K, Pors Y, Guivarc'h A, Mustroph A, Chriqui D, Grimm B, Schmulling T (2008) Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. J Exp Bot **59:** 2659-2672

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmulling T (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15:** 2532-2550

Werner T, Schmülling T (2009) Cytokinin action in plant development. Current Opinion in Plant Biology **12:** 527-538

Wu J, Neimanis S, Heber U (1991) Photorespiration Is More Effective Than the Mehler Reaction in Protecting the Photosynthetic Apparatus against Photoinhibition. Botanica Acta **104:** 283-291

Wuyts N, Palauqui J-C, Conejero G, Verdeil J-L, Granier C, Massonnet C (2010) High-contrast three-dimensional imaging of the Arabidopsis leaf enables the analysis of cell dimensions in the epidermis and mesophyll. Plant Methods **6:** 17

Zubo YO, Yamburenko MV, Selivankina SY, Shakirova FM, Avalbaev AM, Kudryakova NV, Zubkova NK, Liere K, Kulaeva ON, Kusnetsov VV, Borner T (2008) Cytokinin stimulates chloroplast transcription in detached barley leaves. Plant Physiol **148:** 1082-1093

4

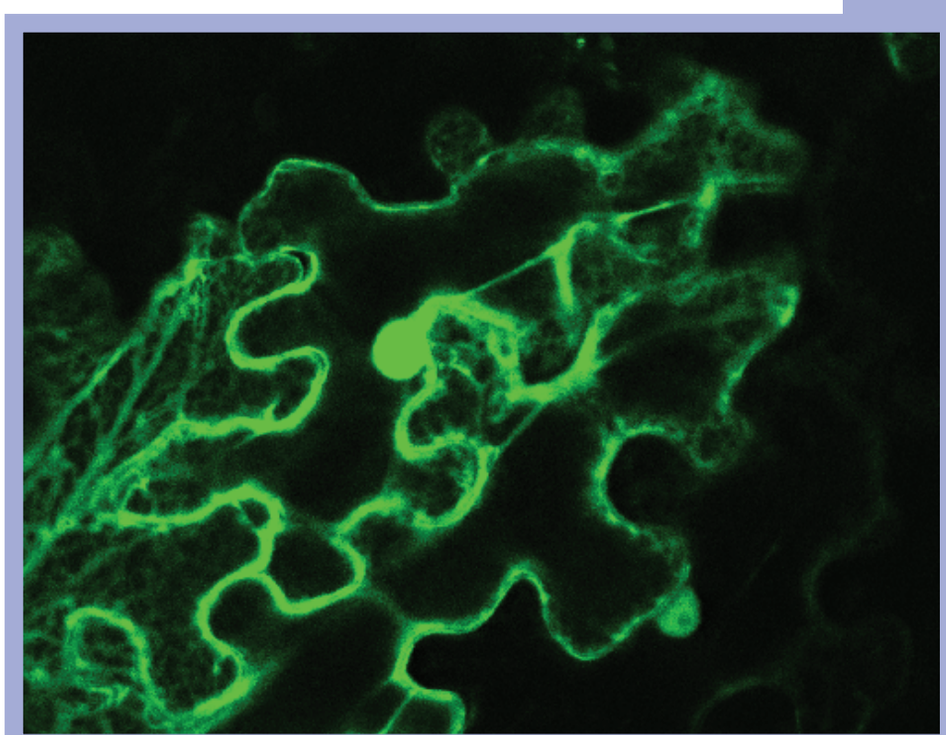


Image: *35S:AN3-EGFP* localization in Tobacco epidermal cells after *Agrobacterium*-mediated leaf infiltration.

Chapter 4: ANGUSTIFOLIA 3 binds Arabidopsis SWI/SNF chromatin remodeling complexes to regulate transcription at the switch of shoot developmental programs

Liesbeth Vercruyssen[1,2], Aurine Verkest[1,2], Nathalie Gonzalez[1,2], Megan Andriankaja[1,2], Soon-Ki Han[3], Rafal Archacki[4], Dominique Eeckhout[1,2], Jelle Van Leene[1,2], Liesbeth De Milde[1,2], Mattias Vermeersch[1,2], Katrien Maleux[1,2], Doris Wagner[3], Andrzej Jerzmanowski[4], Geert De Jaeger[1,2], and Dirk Inzé[1,2]

[1]Department of Plant Systems Biology, VIB, 9052, Ghent, Belgium

[2]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052, Ghent, Belgium

[3]Department of Biology, University of Pennsylvania, PA 19104, Philadelphia, USA

[4]Laboratory of Plant Molecular Biology, University of Warsaw, 02-106, Warsaw, Poland

L.V. wrote the manuscript and conducted the majority of the experiments, except for the tandem affinity purifications (TAP), co-immunoprecipitations (CoIP) and chromatin immunoprecipitations (ChIP). TAP was done by A.V., D.E., J.V.L., and G.D.J, CoIP by A.V., and ChIP by S.H. and D.W. R.A. contributed *35S:BRM* lines and valuable insight. M.A. contributed AGRONOMICS1 tiling array expression data for comparison. L.D.M, M.V. and K.M. gave technical assistance during several experiments. N.G. and D.I. provided guidance and help in correcting the manuscript.

ABSTRACT

The transcription coactivator ANGUSTIFOLIA 3 (AN3) stimulates cell proliferation during Arabidopsis leaf development. Little knowledge is available however on the molecular mechanisms acting upstream and downstream of AN3. Inducible nuclear localization of AN3 during initial leaf growth allowed identification of downstream target genes including important transcriptional regulators. *GROWTH REGULATING FACTORS GRF5*, *GRF6* and *GRF3* and *CYTOKININ RESPONSE FACTOR 2* (*CRF2*) were specifically upregulated already two hours after AN3 activation, and also *HOMEOBOX 33* (*HB33*), *HECATE 1* (*HEC1*), and *CONSTANS-LIKE 5* (*COL5*) were rapidly induced, while *ARABIDOPSIS RESPONSE REGULATOR 4* (*ARR4*) was repressed. AN3 interacts with GRF5 and we propose that together they regulate *HB33* and *HEC1* transcription.

Furthermore, we set out to isolate protein complexes associated with AN3 by tandem affinity purification (TAP), which resulted in the identification of chromatin remodeling complexes from plants. AN3 is shown to be part of SWITCH/SUCROSE NONFERMENTING (SWI/SNF) complexes formed around the ATPases BRAHMA (BRM) or SPLAYED (SYD), including SWI3C and/or SWI3D, SWP73A or SWP73B and ARP4 and ARP7, pointing to a role of AN3 as an interacting protein to guide the chromatin remodeling complex towards the DNA.

Moreover, we demonstrate that expression of *AN3* and its target genes *GRF5*, *HB33*, *HEC1*, *CRF2* and *COL5* is dependent on BRM and that BRM is recruited to the promoter of *HEC1*. In addition, overexpression of *SWI3C* and *BRM* increases leaf size, underlining the importance of chromatin dynamics for growth regulation. Our results place the SWI/SNF-AN3 module as a major player at the transition from cell proliferation to cell expansion in a developing leaf as well as at the transition from vegetative to reproductive development.

INTRODUCTION

When an organism develops, transcription needs to be tightly controlled at the spatial and temporal level, which is accomplished by the combinatorial control of transcription factors, transcriptional coactivators and corepressors, the general transcription machinery, and chromatin modifications (Maston et al., 2006). DNA-binding transcription factors often recruit transcription coactivators and they both promote transcription in similar ways, such as by stimulating general complex formation around RNA polymerase II, or by recruiting chromatin remodellers (Maston et al., 2006).

After Arabidopsis (*Arabidopsis thaliana*) seeds have germinated, new leaves arise from the shoot apical meristem (SAM) as rod-like primordia that develop into mature leaves with adaxial/abaxial and proximodistal polarity. Initially, leaf primordia contain only dividing cells, and this proliferation phase is followed by the transition phase that forms a bridge to the cell expansion phase, where cells exit the mitotic cell cycle and start differentiation (Donnelly et al., 1999; Beemster et al., 2005; Andriankaja et al., 2012). During the transition phase, dividing and expanding cells coexist in the basal and apical

leaf parts, respectively, and the boundary between them, termed the cell cycle arrest front, establishes rapidly at the beginning and disappears abruptly at the end of the transition phase (Kazama et al., 2010; Andriankaja et al., 2012; Gonzalez et al., 2012). Another crucial developmental transition during the Arabidopsis life cycle is the switch from vegetative to reproductive growth when the SAM starts to generate inflorescences and flowers (Srikanth and Schmid, 2011).

ANGUSTIFOLIA 3 (AN3)/GRF-INTERACTING FACTOR 1 (GIF1) is a member of the GIF family of transcriptional coactivators, along with GIF2 and GIF3, with a key role in Arabidopsis shoot development (Kim and Kende, 2004; Horiguchi et al., 2005; Lee et al., 2009). Ectopic expression of *AN3*, *GIF2* and *GIF3* increases leaf size due to an increase in cell number (Horiguchi et al., 2005; Lee et al., 2009). On the other hand, loss of AN3 function results in smaller and narrower leaves with less cells (Kim and Kende, 2004; Horiguchi et al., 2005), because of a premature loss of mitotic activity at a specific stage during primordium outgrowth (Horiguchi et al., 2011). Whereas *gif2* and *gif3* leaves are almost identical to wild-type leaves, double and triple *gif* mutations synergistically reduce cell number, revealing their overlapping and redundant functions (Lee et al., 2009). In addition, *an3*, *gif2* and *gif3* mutation causes a synergistic decrease in flowering time and defects in floral organ formation (Lee et al., 2009).

GIFs, as their name reveals, were first identified by the interaction of AN3 with GROWTH REGULATING FACTOR 1 (GRF1) (Kim and Kende, 2004), a transcription factor that is part of a family comprising 9 members (GRF1-GRF9) (Kim et al., 2003). Like GIFs, GRFs are positive regulators of leaf cell proliferation since overexpression enhances leaf growth and cell division, as shown for *GRF1*, *GRF2* and *GRF5* (Kim et al., 2003; Horiguchi et al., 2005; Kim and Lee, 2006; Gonzalez et al., 2010; Rodriguez et al., 2010). A reduction in leaf cell number has only been shown for *grf4* and *grf5* single mutants (Horiguchi et al., 2005; Kim and Lee, 2006), whereas functional redundancy becomes apparent from the different double, triple or quadruple combinations of *grf1*, *grf2*, *grf3*, *grf4* or *grf5* mutations that synergistically diminish leaf growth (Kim et al., 2003; Kim and Lee, 2006). Moreover, the delayed flowering and partial sterility of *GRF1*, *GRF2* and *GRF5* overexpressing plants hint at an essential role for GRFs in floral transition and flower development (Kim et al., 2003; our own observations). Because of

their similar functions, their physical interaction and the synergistic defects in leaf size and fertility when *an3* mutation is combined with *grf1*, *grf2* and/or *grf3* mutations (Kim and Kende, 2004), GIFs and GRFs are thought to form a functional transcriptional coactivator/transcription factor complex that affects gene expression for correct lateral organ development.

Molecular and genetic data on up- and downstream components of GIF/GRF signaling are only beginning to emerge. Seven *GRFs* are predicted targets of microRNA396 (miR396), which appears at the tip of young leaf primordia and accumulates during leaf growth to restrict *GRF* expression to the basal part of the leaf (Jones-Rhoades and Bartel, 2004; Liu et al., 2009; Rodriguez et al., 2010; Wang et al., 2011a). *MiR396* expression is in turn promoted by TEOSINTE BRANCHED1/CYCLOIDEA/PCF 4 (TCP4) and elevated levels of TCP4, as well as *miR396* overexpression itself, reduce the transcription of all *GRFs* and *AN3* resulting in smaller leaves with less cells (Liu et al., 2009; Rodriguez et al., 2010; Wang et al., 2011a). Several core cell cycle genes expressed during specific but different phases of the cell cycle, e.g. *CYCB1;1* expression during the G2/M transition and *CYCD3;1* during G1/S, were shown to be downregulated in leaf primordia of *an3* mutants and in *35S:miR396* seedlings (Lee et al., 2009; Rodriguez et al., 2010; Ichihashi et al., 2011; Wang et al., 2011a). Taken together, these data demonstrate that AN3 and the GRFs are commonly regulated to fine-tune cell proliferation at the cell cycle arrest front. A direct regulation of cell cycle genes has not been reported however, and transcript profiling of leaves 1&2 of 7-day-old *an3* seedlings merely showed complex changes in metabolism-related gene expression (Horiguchi et al., 2011), hence the transcriptional responses directly downstream of the AN3/GRF module have yet to be uncovered.

The N-terminal domain of GIF proteins is homologous to the SNH domain of human SYNOVIAL TRANSLOCATION (SYT) (Kim and Kende, 2004; Horiguchi et al., 2005), which was shown to interact with human BRAHMA (BRM) and BRAHMA RELATED GENE (BRG1), the two human SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin remodeling ATPases (Thaete et al., 1999; Nagai et al., 2001; Perani et al., 2003). All GRFs contain a N-terminal QLQ domain that shows homology to the yeast SWI2/SNF2 ATPase (van der Knaap et al., 2000; Kim et al., 2003), strongly suggesting

that GIF transcriptional coactivators and GRF transcription factors promote transcription by recruiting SWI/SNF chromatin remodellers.

Two types of chromatin modifications can be distinguished: covalent histone modifications, and ATP-driven movements of nucleosomes to expose or cover specific DNA sequences. SWI/SNF complexes belong to the latter category and are high molecular weight complexes consisting of core and other subunits and variable, often tissue specific associated proteins. The complex subunits are conserved between fungi, animals and plants and are assembled from different combinations of SWI3 proteins, SWI/SNF ASSOCIATED PROTEINs 73 (SWP73s), ACTIN RELATED PROTEINS (ARPs) and/or actin around a central ATPase and a SNF5-like protein (Jerzmanowski, 2007; Kwon and Wagner, 2007). Two types of complexes have been identified from yeast, fruit fly and mammals, each defined by the presence of signature proteins. The first type is named SWI/SNF in yeast, BAP in flies and BAF in humans, and they are all characterized by the presence of one or more AT-RICH INTERACTION DOMAIN (ARID) -like proteins, termed yeast SWI1, fly OSA and human ARID1A/B. The second type of complex is named yeast RSC, fly pBAP and human pBAF, and contains polybromo-like proteins, respectively termed RSC1/2/4, polybromo and BAP170 (Mohrmann and Verrijzer, 2005; Middeljans et al., 2012). Four ATPases were described in Arabidopsis (BRM, SPLAYED (SYD), CHR12 and CHR23); four SWI3 proteins (SWI3A-D); two SWP73 proteins (SWP73A and B); two ARPs (ARP4 and 7) predicted to belong to SWI/SNF complexes; and one homolog of SNF5, termed BUSHY (BSH) (The Chromatin Database, www.chromdb.org; Meagher et al., 2005; Jerzmanowski, 2007; Kwon and Wagner, 2007).

Mammalian SWI/SNF complexes are essential for balancing self-renewal of cells with lineage specific cell differentiation, which is reflected in the tumor suppressor activity of various complex members, and was shown to depend on the exchange of paralogous, often tissue specific, subunits that can bind the complexes (Wilson and Roberts, 2011). Although no SWI/SNF complexes have been isolated from plants and only a few associating proteins were identified, genetic analysis has proven their importance in transcriptional regulation of key developmental processes. Mutation of BRM and SYD results in severely dwarfed plants that have reduced leaf and stem size, perturbed

flowering time and inflorescence and flower development, often leading to sterility (Wagner and Meyerowitz, 2002; Farrona et al., 2004; Hurtado et al., 2006; Archacki et al., 2009). Similar processes are affected in *swi3c* and *swi3d* mutants and *SWI3B* silenced plants, whereas loss of function of SWI3B and SWI3A is embryo lethal (Zhou et al., 2003; Sarnowski et al., 2005; Archacki et al., 2009). Likewise, loss of ARP7 function is embryolethal, while downregulation of *ARP7* and *ARP4* results in pleiotropic phenotypes (Kandasamy et al., 2005a; Kandasamy et al., 2005b). In addition, reduced shoot growth from knockdown of *BSH* and *SWP73B* were reported (Brzeski et al., 1999; Crane and Gelvin, 2007).

Molecular data start to accumulate that strengthen many of the above described phenotypic observations, resulting from mutations in the genes encoding subunits of the SWI/SNF complexes. For example, leaves develop asymmetrically, and adaxial fate is in part promoted by *FILAMENTOUS FLOWER* (*FIL*), a *YABBY* gene family member whose expression is reduced by mutation of *SYD* (Eshed et al., 2004). Likewise, SYD and/or BRM and/or SWI3 proteins were demonstrated to be involved in the regulation of transcription during cotyledon boundary establishment (Kwon et al., 2006), SAM maintenance (Kwon et al., 2005; Han et al., 2008), leaf primordium initiation (Su et al., 2006), vegetative growth by repression of genes encoding seed storage proteins (SSPs) (Tang et al., 2008), the transition to flowering (Sarnowski et al., 2002; Wagner and Meyerowitz, 2002; Farrona et al., 2004; Su et al., 2006; Farrona et al., 2011) and during the expression of homeotic genes when flowers develop (Wagner and Meyerowitz, 2002; Sarnowski et al., 2005; Hurtado et al., 2006; Su et al., 2006; Farrona et al., 2007; Wu et al., 2012). Thus, virtually all aspects of plant development are controlled by SWI/SNF chromatin remodeling, and different complex compositions can have overlapping but also unique targets (Bezhani et al., 2007; Wu et al., 2012). However, direct transcriptional regulation by binding of SYD and/or BRM to the promoter has only been shown for *WUSHEL* (*WUS*) in the SAM, for *APETALA 3* (*AP3*) and *AGAMOUS* (*AG*) during floral whorl formation and for certain *SSPs* (Kwon et al., 2005; Tang et al., 2008; Wu et al., 2012).

Here, we identify important transcriptional regulators that are controlled by AN3, extending the network downstream of the GIF/GRF module. We also report the

identification of Arabidopsis SWI/SNF complexes that are associated with AN3 and provide evidence that chromatin remodeling activity is involved in transcriptional regulation of downstream AN3 targets, suggesting that AN3 functions to recruit SWI/SNF complexes during leaf development.

RESULTS

Induction of AN3 activity enhances leaf growth and *CYCB1;1* expression

To gain insight in the molecular pathways downstream of AN3, plants containing an inducible gain-of-function construct, *35S:AN3-GR*, hereafter designated *AN3-GR*, were generated. Fusion to the Rat Glucocorticoid Receptor (GR) domain retains nuclear proteins in the cytoplasm via association with heat shock proteins (Picard et al., 1988). Addition of dexamethasone (DEX), a glucocorticoid hormone, releases the heat shock proteins, thereby exposing the nuclear localization signals on the GR domain, resulting in translocation of AN3-GR to the nucleus and activation of the downstream transcriptional responses.

Wild-type Columbia-0 (Col-0) and *CYCB1;1:D-Box-GUS-GFP* (*CYCB1;1:DB-GUS*) plants (Eloy et al., 2011) were transformed with the *AN3-GR* construct and independent homozygous lines were obtained. Without DEX application, the size of individual *AN3-GR* leaves at 21 days after stratification (DAS) was indistinguishable from wild-type and *CYCB1;1:DB-GUS* leaf size (Fig. 1, A-C). Growth of *AN3-GR* plants on 25 µM DEX from germination onwards led to the development of larger cotyledons and leaves 1&2, compared to mock treated transformants and DEX treated control plants (Fig. 1, A-C). This phenotype is reminiscent of plants overexpressing *AN3* (Horiguchi et al., 2005), confirming the functionality of the construct.

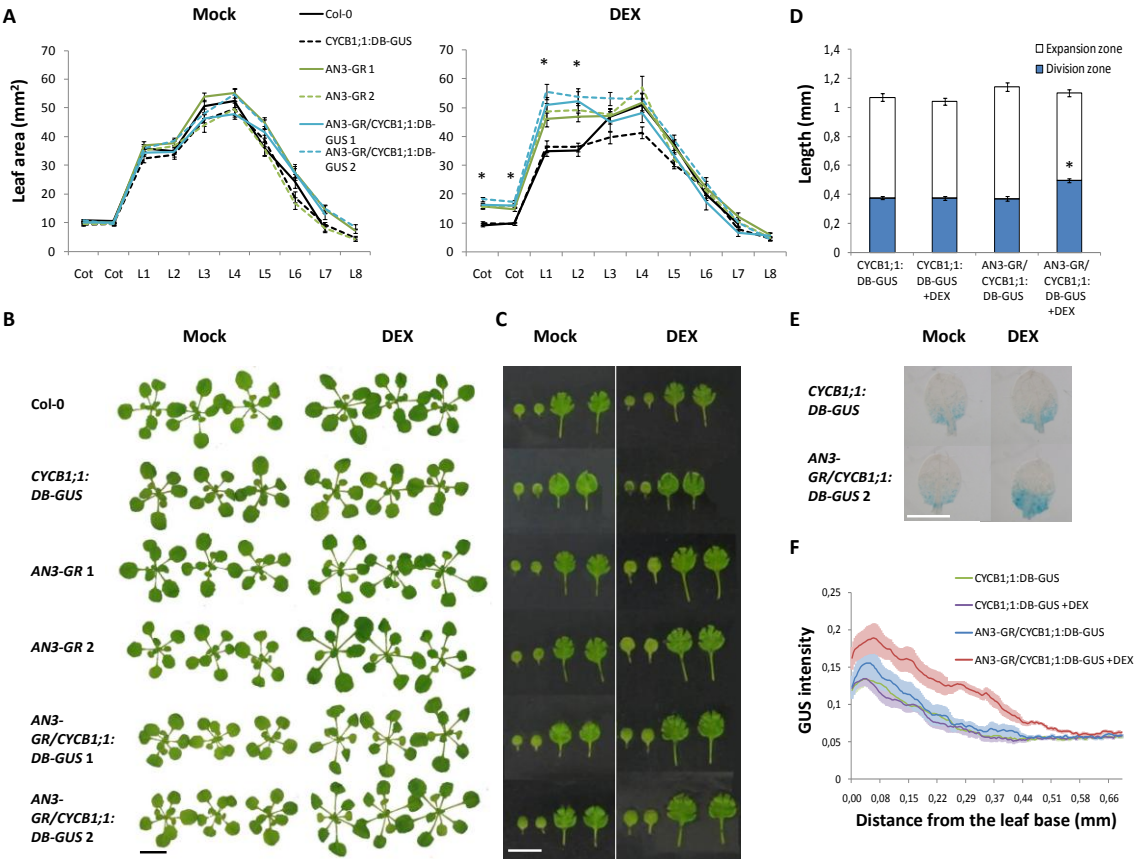


Figure 1. Induction of AN3 activity enhances leaf growth and *CYCB1;1* expression.

A, Leaf area of cotyledons (Cot) and leaves 1 to 8 (L1-L8), measured from leaf series of 21-day-old plants, grown on control medium (Mock) or medium supplemented with 25 µM DEX. Error bars indicate SE ($n \geq 12$). * All significantly different from the wild type (Col-0) ($P < 0.01$, Student's *t* test). B, Rosettes, and C, cotyledons and leaves 1&2 of 21-day-old plants, grown on mock medium or medium with 25 µM DEX. Scale bars: 10 mm. D-F, GUS staining of *CYCB1;1:DB-GUS* and *AN3-GR/CYCB1;1:DB-GUS* leaves 1&2. Plants were grown for 9 days on control medium and subsequently transferred to medium supplemented with 0 or 10 µM DEX for 24h. D, GUS-stained and non-stained regions, indicating the division and expansion zones, respectively, measured along the length of the leaf. Error bars are SE ($n \geq 22$). * Significantly different from DEX-treated control plants ($P < 0.01$, Student's *t* test). E, Leaves 1&2 were mounted on slides for picture taking. Scale bar: 1 mm. F, GUS staining was measured with Image J in a defined area along the leaf length (see materials and methods). Error bars are SE ($n \geq 18$).

In the *CYCB1;1:DB-GUS* transgenics, the *CYCB1;1* promoter restricts GUS expression to the G2/M transition, while the presence of the D-box ensures rapid post-translational degradation of GUS, allowing for quantitative analysis of mitotic activity in developing plants (Colón-Carmona et al., 1999). *AN3-GR/CYCB1;1:DB-GUS* plants were grown for 9 DAS and subsequently transferred to medium supplemented with 10 µM DEX for 24h, after which the first leaves were analyzed for GUS staining. At this stage, mitotic activity is restricted to the basal part in *CYCB1;1:DB-GUS* leaves (Fig. 1, D and E) (Donnelly et al., 1999; Beemster et al., 2005). Induction of AN3 activity extended the region of GUS staining measured along the length of the leaf while total leaf length was unaffected by 24h DEX treatment (Fig. 1, D and E). In addition, the GUS intensity in the stained region was increased in *AN3-GR/CYCB1;1:DB-GUS* leaves compared to untreated and *CYCB1;1:DB-GUS* leaves (Fig. 1, E and F). The early loss of mitotic activity and the lower maximum proliferation rate in the *an3* mutant (Lee et al., 2009; Horiguchi et al., 2011) are consistent with our findings that indicate a function for AN3 in both the duration and the rate of cell proliferation.

Identification of downstream AN3 responses

How does AN3, described to be a transcriptional coactivator, positively regulate leaf cell division? To answer this question, developing first leaves of *AN3-GR* and wild-type plants were subjected to transcript profiling using Affymetrix ATH1 microarrays after transfer to DEX-containing medium for 8 hours at 8 DAS. At this time point, a substantial amount of cells in leaves 1&2 is proliferating, while other cells start transitioning from cell proliferation to cell expansion. Wild-type *AN3* expression is associated with proliferating cells (Horiguchi et al., 2005; Horiguchi et al., 2011; Ichihashi et al., 2011; Wang et al., 2011a) and decreases at 8 DAS, resulting in a stronger difference in transcriptional activity upon steroid induction of AN3 in *AN3-GR* leaves. Activation of AN3 function resulted in 119 genes whose expression was induced, while expression of 48 genes was reduced, including 11 and 5 transcription factors, respectively, when a false discovery rate < 0.05 was applied (Table 1; Supplemental

Table 1). Strikingly, 4 members of the *GRF* family were upregulated: *GRF5*, *GRF6*, *GRF3* and *GRF8*.

Functional enrichment analysis for MapMan categories with PageMan (Usadel et al., 2006) revealed an overrepresentation amongst the upregulated genes of categories including RNA processing and RNA regulation of transcription, DNA synthesis and chromatin structuring, amino acid activation pseudouridylate synthesis, ribosomal protein synthesis, ABC1 family proteins and pentatricopeptide repeat (PPR) -containing proteins (Supplemental Fig. 1, A). In addition, overrepresentation analysis of subcellular localization with PLAZA (Proost et al., 2009) uncovered the presence of transcribed proteins predominantly in the nucleus, the nucleolus, and the intracellular organelle lumen (Supplemental Fig. 1, C). Ribosome biogenesis and assembly starts in the nucleolus (Fatica and Tollervey, 2002; Zemp and Kutay, 2007), while PPR proteins are involved in RNA processing in the mitochondria and chloroplasts (Schmitz-Linneweber and Small, 2008). The downregulated genes were enriched in categories with sulfur-containing secondary metabolism, minor carbohydrate and C1 metabolism, peptide transport and miscellaneous processes including protease inhibitor/seed storage/lipid transfer proteins (LPT) (Supplemental Fig. 1, B). All together, this suggests a role for AN3 in the regulation of the general processes that sustain the high metabolic rate of dividing cells.

Comparison of the differentially expressed genes with recently published transcriptome sets of leaf 3 from day 8 to day 13, covering the subsequent phases from proliferation to expansion (Andriankaja et al., 2012), revealed a strong significant overlap between the AN3-upregulated genes and the genes whose expression goes down between days 9 and 10 (Fig. 2, A), concomitant with a sharp transition from cell proliferation to expansion. Both datasets are enriched for similar functional categories, namely RNA processing and RNA regulation of transcription, DNA synthesis and chromatin remodeling, and ribosomal protein synthesis. An albeit smaller but significant overlap was also found between the genes downregulated after AN3-GR induction and the genes upregulated during leaf 3 development between days 9 and 10 (Fig. 2, B), and common enriched categories include secondary metabolism and peptide transport. Moreover, expression of most of the genes in both overlaps gradually decreases or increases, respectively, during

leaf 3 development between days 8 and 13 (Supplemental Fig. 2, A and B), confirming that AN3 functions in activating gene transcription that favors cell proliferation while inhibiting expression of genes that promote differentiation.

In addition, the intersection was analyzed with microarray data from *an3* mutant leaves 1&2 (Horiguchi et al., 2011) and *35S:GRF5* rosettes at stage 1.03 (Gonzalez et al., 2010). Although the number of genes in the intersections was small, upregulated genes from DEX treated *AN3-GR* leaves significantly overlapped with genes regulated in the same direction in *35S:GRF5* plants and in the opposite direction in *an3* mutant plants, and vice versa (Fig. 2, A and B). Genes with higher expression in both *AN3-GR* and *35S-GRF5* leaves encode the HOMEOBOX 33 transcription factor (HB33) and the basic helix-loop-helix (bHLH) transcriptional regulator HECATE 1 (HEC1) (Table 1).

Table 1. Transcription factors differentially expressed after induction of AN3 activity

AGI Code	Annotation	FC	P-value	*35S:GRF5*	*an3*	WT 9-10 DAS
AT5G28640	AN3 (ANGUSTIFOLIA 3)	8,26	0,00031		DOWN	
AT5G57660	COL5 (CONSTANS-LIKE 5)	3,38	0,04161		UP	
AT5G67060	HEC1 (HECATE 1)	2,48	0,04199	UP		
AT3G13960	GRF5 (GROWTH-REGULATING FACTOR 5)	2,32	0,01151	UP		DOWN
AT1G75240	HB33 (HOMEOBOX PROTEIN 33)	1,94	0,01975	UP		DOWN
AT2G06200	GRF6 (GROWTH-REGULATING FACTOR 6)	1,92	0,03288			
AT4G39780	AP2 domain-containing transcription factor, putative	1,55	0,04592			
AT2G36400	GRF3 (GROWTH-REGULATING FACTOR 3)	1,54	0,04199	DOWN		DOWN
AT2G42870	PAR1 (PHY RAPIDLY REGULATED 1)	1,54	0,04161			
AT4G24150	GRF8 (GROWTH-REGULATING FACTOR 8)	1,51	0,04865			
AT4G23750	CRF2 (CYTOKININ RESPONSE FACTOR 2)	1,46	0,04161			
AT1G51700	ADOF1 (Dof zinc finger protein)	-1,65	0,04161			
AT1G71030	MYBL2 (MYB-LIKE 2)	-1,64	0,03820		UP	
AT5G47640	NF-YB2 (NUCLEAR FACTOR Y, SUBUNIT B2)	-1,52	0,03288			
AT1G10470	ARR4 (RESPONSE REGULATOR 4)*	-1,49	0,05260		UP*	
AT1G28370	ERF11 (ERF DOMAIN PROTEIN 11)	-1,48	0,04085			
AT5G66070	zinc finger (C3HC4-type RING finger) family protein	-1,43	0,04536			

Affymetrix ATH1 transcript profiles of *AN3-GR* leaves 1&2 compared to Col-0 leaves 8h after DEX treatment in three biological replicates each. Differentially expressed transcription factors with P-value < 0.05 are shown. Upregulated (no shading) and downregulated (grey shading) genes are ordered according to Fold Change (FC). Columns 5, 6 and 7 indicate the intersection with publicly available microarray datasets of *35S:GRF5* shoots (Gonzalez et al., 2010), *an3* leaves 1&2 (Horiguchi et al., 2011) and wild-type (WT) Col-0 leaf 3 between days 9 and 10 (Andriankaja et al., 2012) (see Fig. 3). DOWN: downregulated. UP: upregulated. * The P-value of *ARR4* is not < 0.05, but the gene is upregulated in *an3* and independently confirmed, as shown in Supplemental Fig. 5.

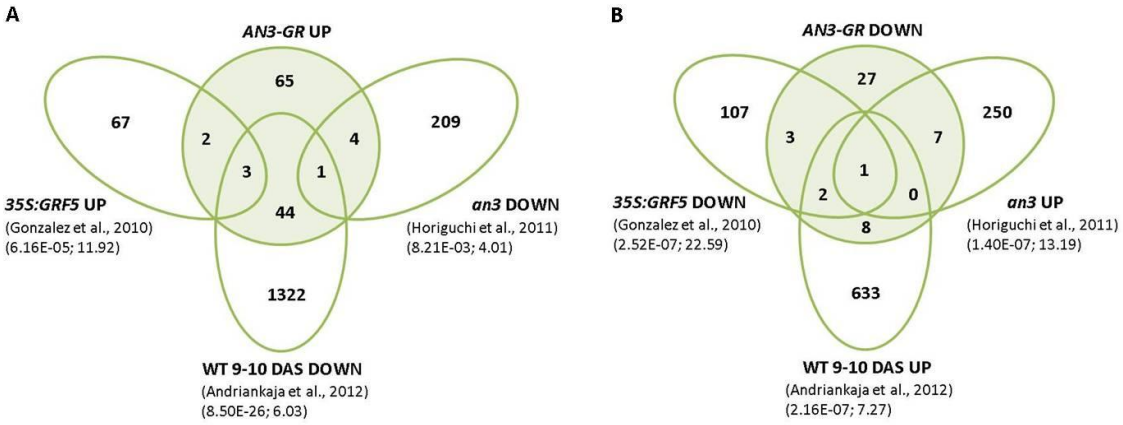


Figure 2. Comparison of the differentially expressed genes after AN3 induction with publicly available microarray datasets.

A, Intersection of *AN3-GR* upregulated genes with genes upregulated in *35S:GRF5* and downregulated in *an3* and between days 9 and 10 during wild-type (Col-0) leaf 3 development. B, Intersection of *AN3-GR* downregulated genes with genes going down in *35S:GRF5* and up in *an3* and during wild-type leaf 3 development between days 9 and 10. The numbers between brackets indicate P-value, showing significant enrichment ($P < 0.01$, Fisher exact test) and fold enrichment, respectively, for each overlap.

AN3 regulates the expression of *CRF2, HEC1, HB33, COL5* and *GRF* transcription factors

During eight-hour treatment with DEX, several cycles of transcription-translation lead to the up- or downregulation of genes that are most likely not directly controlled by AN3. To identify rapidly transcriptionally regulated targets, a time course experiment was conducted, in which RNA levels were quantified with qRT-PCR 1, 2, 4 and 6 hours after transfer of *AN3-GR* and wild-type plants to DEX containing medium. The transcripts of all differentially expressed transcription factors were analyzed, as well as the remaining *GRFs*, to investigate the preference of AN3 for regulation of certain *GRFs* and because *GRF4* and *GRF9* are not represented on the ATH1 array.

GRF5, *GRF6* and *GRF3* were induced significantly from two hours onwards (Fig. 3, A). The transcript levels of *GRF7* and *GRF8* were in general low and variable in the first leaves, and not significantly affected by DEX treatment. Also, the expression of the other *GRFs* was not markedly changed (Fig. 3, B), in accordance with the microarray data, suggesting that AN3 directly and specifically activates the transcription of *GRF5*, *GRF6*

and *GRF3* during early leaf development. In addition, *AN3* expression itself seems to be reinforced 6 hours after treatment.

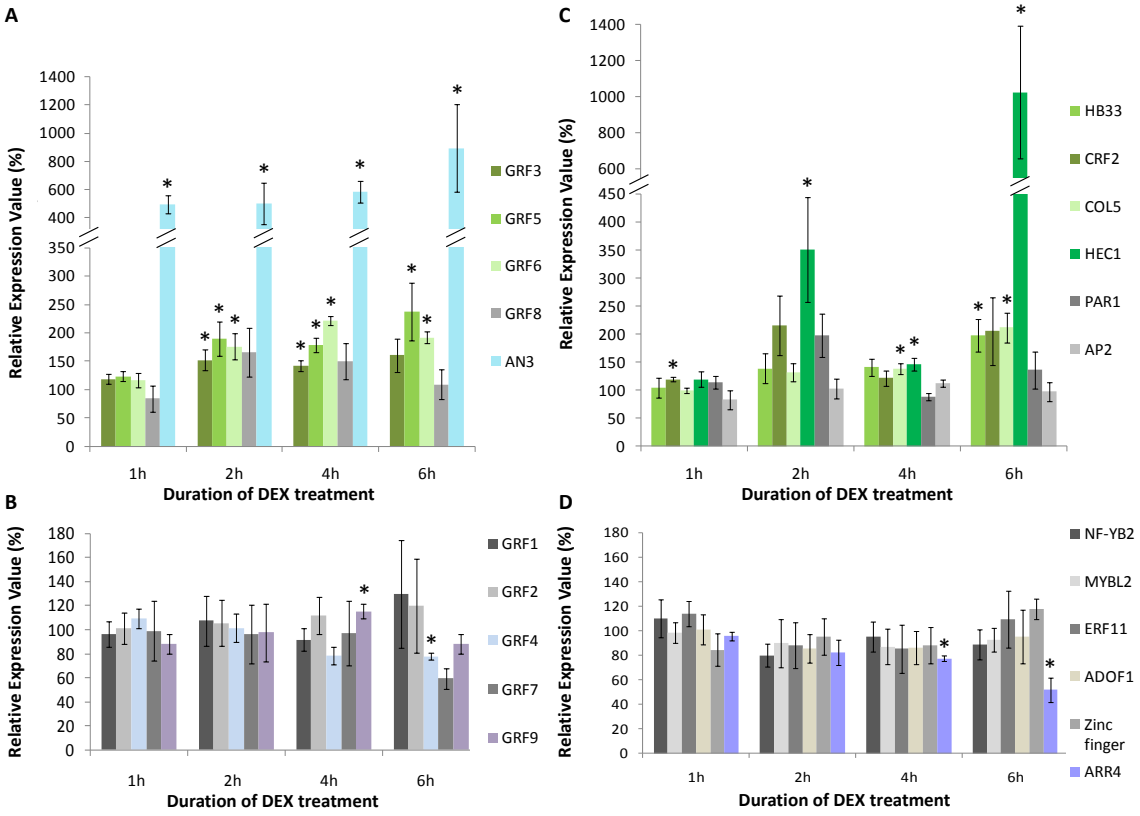


Figure 3. Identification of AN3 targets by time course analysis of expression levels.

A-D, Wild-type (Col-0) and *AN3-GR* plants were grown for 8 days and subsequently transferred to medium supplemented with 5 µM DEX for 1h, 2h, 4h and 6h. Expression levels were determined by qRT-PCR and normalized to DEX-treated wild-type expression levels which are set at 100% for each time point. Error bars are SE (n = 3). * Significantly different from DEX-treated wild-type plants ($P < 0.1$, Student's *t* test). A, Transcript levels of *AN3* and the *GRFs* identified as differentially expressed in the microarray analysis 8h after DEX treatment (Table 1). B, Transcript levels of remaining *GRFs*. C, Transcript levels of the other transcription factors upregulated in *AN3-GR* 8h after DEX treatment (Table 1). D, Expression levels of the transcription factors downregulated in *AN3-GR* 8h after DEX treatment (Table 1).

The expression of four other transcription factors was induced early (Fig. 3, C): *CYTOKININ RESPONSE FACTOR 2* (*CRF2*) was significantly upregulated already one hour after transfer to DEX, *HEC1* after two hours, and *HB33* and *CONSTANS-LIKE 5* (*COL5*) were induced after four hours (Fig. 3, C). *HEC1* transcript levels are very low in wild-type leaves 1&2, explaining the high normalized expression values and variability compared to DEX-treated *AN3-GR* leaves.

None of the significantly downregulated transcription factors appeared to be repressed earlier than eight hours (Fig. 3, D), which led to testing the expression of A-type *ARABIDOPSIS RESPONSE REGULATOR 4* (*ARR4*), despite its FDR-corrected P-value of 0.0526 (Table 1). We were particularly interested in *ARR4* because this gene was found to be upregulated in the *an3* mutant and downregulated in *35S:GRF5* plants, as validated with a nCounter nanostring platform (Supplemental Fig. 3). *ARR4* transcript levels were significantly lower in *AN3-GR* leaves four and six hours after induction compared to WT (Fig. 3, D), confirming its repression by AN3.

In conclusion, the time course experiment identified *GRF3*, *GRF5*, *GRF6*, *CRF2*, *HEC1*, *HB33*, *COL5*, and *ARR4* as genes whose expression is rapidly changed upon AN3 activation (Fig. 3), possibly rendering them direct targets of AN3 transcriptional regulation during leaf formation.

AN3 and GRF5 physically and genetically interact

We demonstrated that AN3 promotes the expression of particular *GRFs*, including *GRF5*. Similar leaf growth phenotypes result from overexpression of *AN3* and *GRF5* (Horiguchi et al., 2005), characterized by an increased area of cotyledons and leaves 1&2 at 21 DAS (Fig. 4, A and B), whereas mutation of *an3* reduces leaf growth more drastically compared to *grf5* loss of function (Kim and Kende, 2004; Horiguchi et al., 2005) (Fig. 4, A and B). By co-immunoprecipitation (CoIP), we confirmed the previously reported direct binding of AN3 with GRF5 (Supplemental Fig. 4) (Kim and Kende, 2004; Horiguchi et al., 2005). To further investigate the genetic interaction between the GRF5 transcription factor and the AN3 coactivator, *AN3* was overexpressed in *grf5* plants, and reciprocally *35S:GRF5* was introduced in the *an3* mutant.

At 21 DAS, individual rosette leaves of *35S:AN3/grf5* plants were comparable in size to the leaves of *35S:AN3* plants (Fig. 4, A and B), indicating that ectopic expression of *AN3* not only compensated for loss of *grf5* function, but also increases leaf size similarly, despite the absence of GRF5. Thus, *AN3* does not seem to need GRF5 to produce the phenotype when overexpressed, suggesting that they might not function together. However, given their binding, redundancy amongst GRF family members that substitute GRF5 function and are activated by AN3 provides a reasonable explanation.

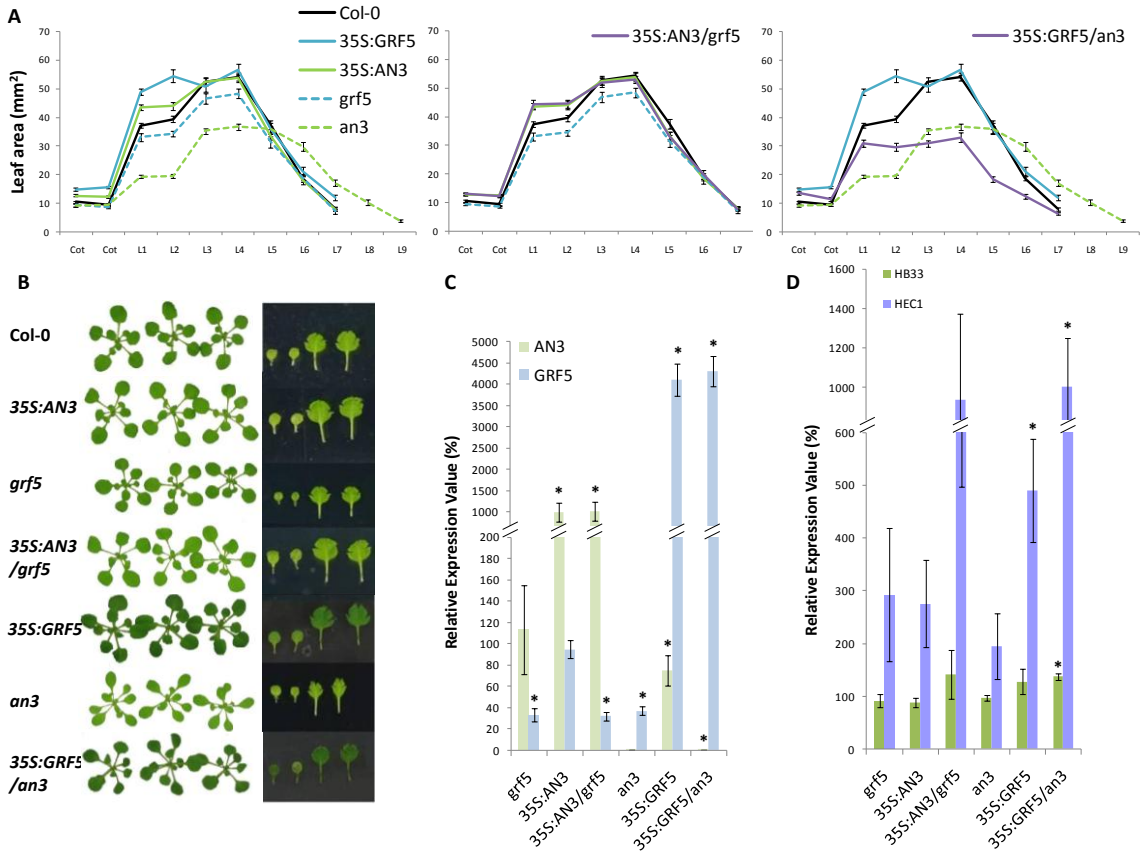


Figure 4. AN3 and GRF5 genetically interact.

A, Leaf area of cotyledons (Cot) and leaves 1 to 9 (L1-L9), measured from leaf series of 21-day-old plants. Error bars indicate SE ($n \geq 14$). B, Rosettes, cotyledons and leaves 1&2 of 21-day-old plants. C and D, Relative expression levels in transgenic rosettes of *AN3* and *GRF5* (C) and *HB33* and *HEC1* (D) at 12 DAS, for each gene normalized to wild-type (Col-0) expression level which is set to 100%. Error bars are SE ($n = 3$). * Significantly different from Col-0 ($P < 0.05$, Student's *t* test).

Cotyledons and leaves 1&2 of *35S:GRF5/an3* plants were intermediate in size between the size of single *35S:GRF5* and *an3* cotyledons and leaves 1&2. Leaves 3 and 4 are equal in size to leaves 1&2 and the younger leaves gradually become smaller, a leaf series profile more reminiscent of that of *35S:GRF5* plants (Fig. 4, A and B). Furthermore, the leaves of *35S:GRF5* plants are darker green, a feature that is still observed in *35S:GRF5/an3* plants (Fig. 4, B). On the one hand this suggests that AN3 is not essential for GRF5 function. Either GIF2 and GIF3 can substitute for AN3, but only partially due to the more profound role of AN3 (Lee et al., 2009), or GRF5 associates with other yet to be discovered transcriptional coactivators. On the other hand it shows that *GRF5* overexpression can only partially rescue loss of an3 function, suggesting that other and more GRFs are needed for proper AN3 signaling, and/or that other transcription factors interact with AN3 to activate GRF-independent downstream responses.

The need for other GRFs to compensate loss of *an3* function is strengthened by the observation that *GRF5* and *GRF6* are downregulated in *an3* seedlings, while *GRF3* and *GRF8* expression is unchanged (Fig. 4, C; Supplemental Fig. 5). Combination of *35S:GRF5* with *an3* further reduces *GRF6* expression, probably by means of a negative feedback mechanism, since *GRF3* and *GRF8* are also downregulated when *GRF5* is overexpressed (Table 1; Supplemental Fig. 5). Thus, although *GRF5* expression is strongly increased in *35S:GRF5/an3* plants, expression of *GRF6* and possibly other specific *GRFs* is severely reduced, probably preventing, at least in part, the complementation of the *an3* mutant phenotype.

Next, expression levels of remaining putative direct target transcription factors of AN3 were analyzed in *35S:AN3/grf5* and *35S:GRF5/an3* plants. No consistent differences in transcription could be observed for *CRF2* and *COL5* (data not shown), supporting their transcriptional regulation by AN3 with the aid of other GRFs or other transcription factors. Microarray analysis revealed that *HB33* and *HEC1* are upregulated in both *AN3-GR* and *35S:GRF5* seedlings (Table 1; Gonzalez et al., 2010), making them promising candidates to be coordinately regulated by the AN3/GRF5 complex. QRT-PCR showed that expression of *HB33* was increased in *35S:GRF5* overexpressing rosettes at 12 DAS, although not significantly, and this increase could still be observed when *an3* was mutated (Fig. 4, D). Similarily, despite the previously mentioned low and variable mRNA

levels of *HEC1* as illustrated by the large error bars, significant increases could only be observed when *GRF5* was ectopically expressed, even in the *an3* mutant background (Fig. 4, D). These observations underline our findings that *HB33* and *HEC1* are most likely under transcriptional control of the AN3/GRF5 complex and are putatively part of the ability of *GRF5* overexpression to partially compensate the decrease in *an3* leaf growth.

In addition, combined overexpression of *GRF5* and *AN3* by crossing of *35S:GRF5* with *RolD:AN3* plants, in which *AN3* expression is controlled by a constitutive promoter from *Agrobacterium rhizogenes*, results in an additive effect on leaf size (Supplemental Fig. 6). This argues against a simplified model with total redundancy amongst GRFs that act downstream of AN3, and favors a model where AN3 has GRF5-independent functions. Moreover, *GRF5* overexpression enhances leaf growth more than *AN3* overexpression (Fig. 4, A and B), suggesting that GRF5 is a limiting factor of the AN3/GRF5 interaction.

AN3 associates with SWI/SNF chromatin remodeling complexes

The existence of domains with homology to chromatin remodeling proteins in AN3 as well as GRF5 brings about the likelihood that AN3 has additional interacting partners, besides GRFs. To investigate this, AN3 was used as a bait for Tandem Affinity Purification followed by Mass Spectrometry analysis (TAP/MS), a powerful method to isolate and identify protein complexes (Van Leene et al., 2007; Van Leene et al., 2008; Van Leene et al., 2011).

C- and N-terminal fusions of AN3 to the GS TAP-tag, containing two protein G IgG-binding sites and a streptavidin-binding peptide, separated by two tobacco etch virus cleavage sites, were expressed under the control of the *35S* promoter in Arabidopsis cell cultures. Eight independent TAP experiments resulted in the isolation of 16 proteins, including AN3 (Table 2). Recently, TAP has been optimized for Arabidopsis seedlings (see materials and methods). The use of six-day-old seedlings expressing TAP-tagged *AN3* from the moderately constitutive *CDKA;1* promoter, confirmed 11 out of the 16 proteins isolated from cell cultures. In addition, eight new preys could be identified (Table 2).

Strikingly, several plant homologs of subunits of SWI/SNF complexes were repeatedly purified from cell culture and seedlings, including SYD, BRM, SWI3C, SWP73B, ARP4 and ARP7. SWI3D and SWP73A were isolated from plants as well. Hence, AN3 interacts with plant SWI/SNF complexes and the presence of two ATPases and two SWP73 proteins suggests that AN3 can be part of at least two different SWI/SNF complexes. Furthermore, GRF3 was copurified, along with another transcription factor, WUSCHEL-RELATED HOMEOBOX13 (WOX13), which is of particular interest as strong *WOX13* expression is observed in leaf primordia (Deveaux et al., 2008), and both transcription factors could provide DNA binding specificity to the chromatin remodeling complexes.

Reverse TAP experiments with SWI3C, SWP73B, and ARP4 (Table 2) allowed the reconstruction of putative SWI/SNF complexes around AN3 and BRM, visualized as a network made in Cytoscape (Shannon et al., 2003) (Fig. 5). The edges do not necessarily represent direct protein-protein interactions, because TAP does not allow for distinction between direct and indirect interactions. Nevertheless, the reciprocal TAPs provide useful information on the architecture of plant SWI/SNF complexes (Table 2; Fig. 5). First, SWI3C TAP-fusion pulled down BRM as a single ATPase, and other SWI3 proteins were absent, confirming the preferred coexistence of SWI3C with BRM (Hurtado et al., 2006; Archacki et al., 2009). Second, TAP with SWP73B yielded ATPase CHR12 on top of BRM, BSH, and all SWI3 proteins, but lacked SWP73A, while both SWP78 proteins were detected by AN3, SWI3C and ARP4 TAP. This indicates that SWP73A and SWP73B are mutually exclusive but show rather low specificity for a certain subunit composition. Third, like SWI3C, ARP4 preferentially occurs with BRM and SWI3C, since no paralogs were identified. Fourth, ARP4 and ARP7 were detected in all experiments, proving their coexistence and last, two unknown proteins encoded by At5g55210 and At5g17510 represent new high confidence plant SWI/SNF interacting proteins given their frequent purification by all four baits (Table 2, Fig. 5).

Table 2. Tandem Affinity Purification with AN3, SWI3C, SWP73B, and ARP4 as baits.

AGI code	Annotation	ChromDB ID	AN3 8 exps	AN3 *planta* 1 exp	SWI3C 5 exps	SWP73B 4 exps	ARP4 4 exps
At2g28290	SYD	CHR3	8	1			
At3g06010	MINU1	CHR12				1	
At2g46020	BRM	CHR2	6	1	5	3	2
At3g17590	BSH	CHE1				4	
At2g47620	SWI3A	CHB1				5	
At2g33610	SWI3B	CHB2				4	
At1g21700	SWI3C	CHB4	4	1	5	4	2
At4g34430	SWI3D	CHB3		1		4	
At3g01890	SWP73A	CHC2		1	5		2
At5g14170	SWP73B	CHC1	4	1	5	5	2
At1g18450	ARP4	ARP4	4	1	5	3	4
At3g60830	ARP7	ARP7	7	1	5	4	4
At1g20670	BRD1	BRD1	1	1	5	5	2
At1g76380	BRD2	BRD2		1	4		2
At1g58025	BRD5	BRD5				1	
At5g55040	BRD13	BRD13		1		2	1
At1g54390	PHD finger protein related	INGF2					1
At5g45600	TAF14B	YDG1					2
AT5G28640	AN3	-	4	1			
AT4G17330	G2484-1	-	6	1			
AT4G16143	IMPA-2	-	5				
AT5G55210	Unknown protein	-	4	1	4	4	1
AT3G06720	IMPA-1	-	4				
AT5G17510	Unknown protein	-	3	1	5	4	2
AT5G53480	Putative importin beta-2	-	4				
AT1G09270	IMPA-4	-	1				
AT1G26570	UGD1	-		1			
AT2G36400	GRF3	-		1			
AT3G22990	LFR	-		1		4	
AT4G35550	WOX13	-	1				
AT5G07980	Dentin sialophosphoprotein-related	-		1			
AT1G47128	RD21	-				4	2
AT5G14240	Thioredoxin superfamily protein	-					2
AT3G03460	Unknown protein	-			1		1
AT4G22320	Unknown protein	-			1	1	
AT4G04740	CPK23	-			1		
AT1G32730	Unknown protein	-				4	
AT1G06500	Unknown protein	-				3	
AT3G18380	Homeobox transcription factor	-				2	
AT3G50000	CKA2	-				1	

TAP was performed on Arabidopsis cell cultures, and in addition for AN3 on Arabidopsis seedlings as indicated in column 5 (*planta*). Column 3 shows the presence in The Chromatin Database (www.chromdb.org). The numbers indicate the amount of experiments in which the protein was identified.

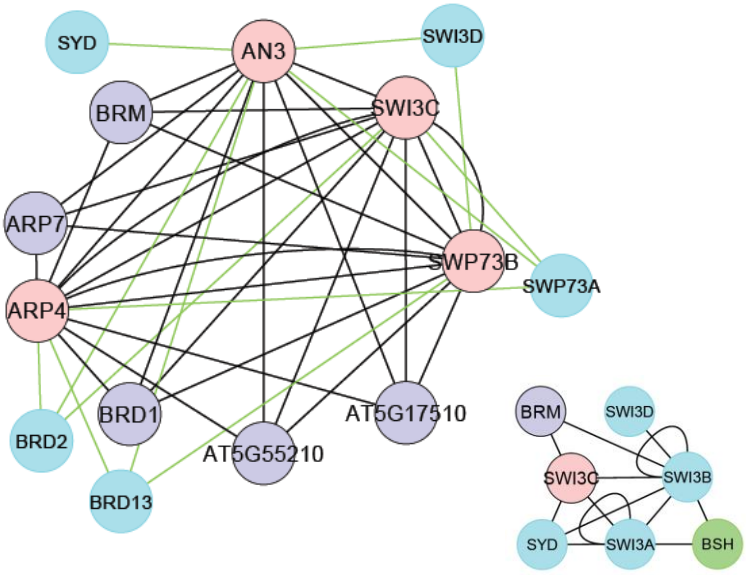


Figure 5. Tandem Affinity Purification reveals interaction of AN3 with SWI/SNF complexes.
Cytoscape (Shannon et al., 2003) protein interaction networks are based on the TAP experiments shown in Table 2. Pink nodes indicate proteins used as bait for the TAP experiments and purple nodes indicate proteins that were pulled down with each of the four baits. Paralogous proteins identified by AN3 TAP are represented by blue nodes. Black edges are used when the proteins were identified by all four baits, and green edges are used for proteins identified by three or less bait proteins. The inset shows the Y 2-H and *in vitro* pull-down interactions between SWI/SNF subunits that were previously reported in literature (Sarnowski et al., 2002; Farrona et al., 2004; Sarnowski et al., 2005;. Hurtado et al., 2006; Bezhani et al., 2007). The single family member protein BSH is depicted by a green node.

Furthermore, three bromodomain proteins (BRD1, BRD2 and BRD13) stand out, because of homology with animal and yeast polybromo proteins, which are signature proteins that define the functional SWI/SNF class. Plants lack proteins with multiple bromodomains (Pandey et al., 2002). Instead, proteins with one bromodomain are postulated to associate and act as functional homologs of animal polybromo proteins (Jerzmanowski, 2007). The reciprocal isolation of BRD1, BRD2 and BRD13 with AN3, ARP4, SWI3C, and SWP73B as baits provides substantial evidence for this hypothesis. In addition, AN3 and SWP73B TAP isolated LEAF AND FLOWER RELATED (LFR), a protein that shows 28% homology to human ARID2/BAF200 (Wang et al., 2009), shown to be a signature protein of pBAF (Polybromo-associated BAF) complexes. Moreover, given the absence of homologs of human ARID1 proteins, which define BAF complexes, in the

interactome, the SWI/SNF complexes around AN3 show more resemblance to animal pBAF complexes, which provides evidence for a similar subdivision in the type of SWI/SNF complexes in plants.

Taken together, we isolated SWI/SNF chromatin remodeling complexes from Arabidopsis, revealing that AN3 resides in pBAF-like complexes composed of BRM *or* SYD, SWI3C *and/or* SWI3D, SWP73A *or* SWP73B, and ARP4 *and* ARP7.

BRM affects expression of *AN3* and its target genes

DEX treatment causes translocation of AN3 to the nucleus, and our data suggest that AN3 binds there to both SWI/SNF complexes and GRFs or other transcription factors, which guide the complexes towards the promoters of the AN3 target genes. To investigate whether remodeling of the chromatin is necessary for proper activation or repression of the genes regulated by AN3, their expression was analyzed in *brm* mutants.

The *brm1* mutant has a T-DNA insert in the first exon, resulting in severe developmental defects, such as small spiral-shaped leaves with downward curling edges (Fig. 6, B) (Hurtado et al., 2006). The *brm3* mutant shows only a mild reduction in leaf growth (Fig. 6, B), because here T-DNA insertion gives rise to a truncated protein only missing the 454 carboxy-terminal amino acids including the bromodomain, which does not seem to interfere with complex assembly (Farrona et al., 2007). Transcript levels of *AN3* and its target genes in the shoots of 12-day-old *brm1*, *brm3* and wild-type seedlings were compared by qRT-PCR (Fig. 6, A and B). Expression of *GRF5*, *COL5* and *CRF2* was reduced in both mutants, significantly in *brm1* and *brm3* for *COL5* but only in *brm1* for *GRF5* and *CRF2*. *HEC1* was significantly downregulated in *brm3* shoots, whereas expression in *brm1* was more variable. In addition, *brm1* and *brm3* showed a comparable reduction in mRNA levels of *AN3* itself. *GRF3*, *GRF6* and *ARR4* were not differentially expressed when *brm* was mutated, whereas *HB33*, in contrast to our expectations, was upregulated (Fig. 6, A). The reduced expression of *GRF5*, *COL5*, *CRF2*, *HEC1* and *AN3* in *brm3* at a developmental stage where shoot growth is similar to WT suggests that a reduction in BRM activity is causing the downregulation, rather than differences in morphology.

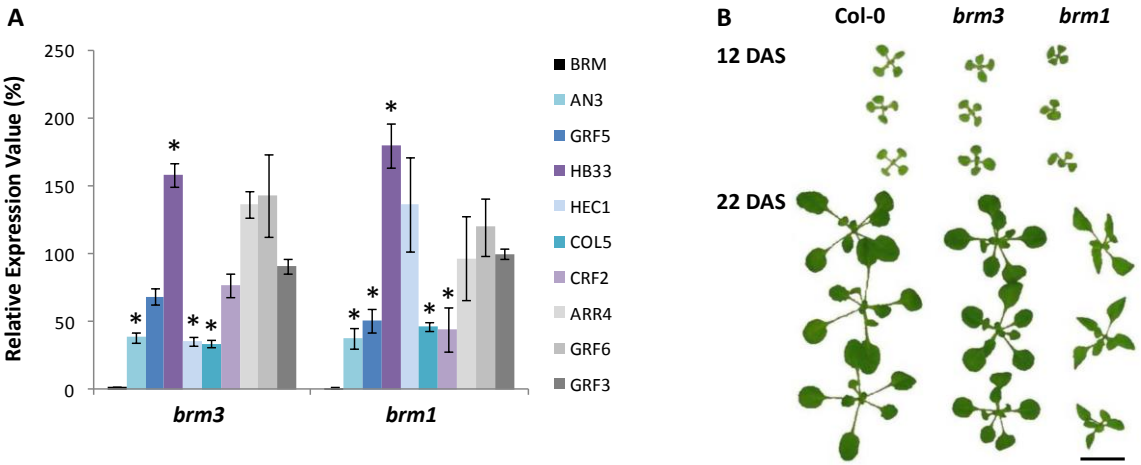


Figure 6. BRM affects expression of *AN3* and its target genes.

A, Relative expression levels determined by qRT-PCR in *brm3* and *brm1* rosettes of 12-day-old plants, for each gene normalized to wild-type (Col-0) expression level which is set to 100%. Error bars are SE (n = 3). * Significantly different from wild-type plants ($P < 0.1$, Student's *t* test). B, Rosettes of wild-type, *brm3* and *brm1* plants at 12 DAS, the stage used for qRT-PCR analysis shown in A, and at 22 DAS (Scale bar: 10 mm).

BRM is present at the promoter of *HEC1*

According to our data, the transcription of *AN3* and genes rapidly regulated by AN3 seems to depend on BRM, most likely because BRM is recruited by AN3 to the promoter regions to move the nucleosomes and thereby modulate the accessibility of cis-regulatory elements. Chromatin immunoprecipitation (ChIP) is a powerful technique used previously to show the presence of BRM at the promoter of genes encoding seed storage proteins (Tang et al., 2008). Also, ChIP allowed the detection of SYD at the promoter of *WUS*, and both BRM and SYD were able to bind to the *AP3* and *AG* regulatory regions (Kwon et al., 2005; Wu et al., 2012). Here, ChIP was used to examine the presence of BRM at the *AN3* promoter and the promoter of selected direct targets. Wild-type control plants and transgenics expressing *HA*-tagged *BRM*, were grown until 9 DAS, chromatin was precipitated with anti-*HA* antibody and enrichment of selected DNA sequences was determined by qPCR. Primers annealing to the promoter or 5' UTR region revealed a

strong enrichment for *HEC1* in *BRM-HA* shoots, while no significant differences were found compared to control plants for *AN3*, *GRF5*, *GRF6*, *GRF3* or *HB33* promoter regions, nor for the heterochromatic *Ta3* control locus (Fig. 7) (Johnson et al., 2002). Thus, BRM physically associates with the promoter of *HEC1*.

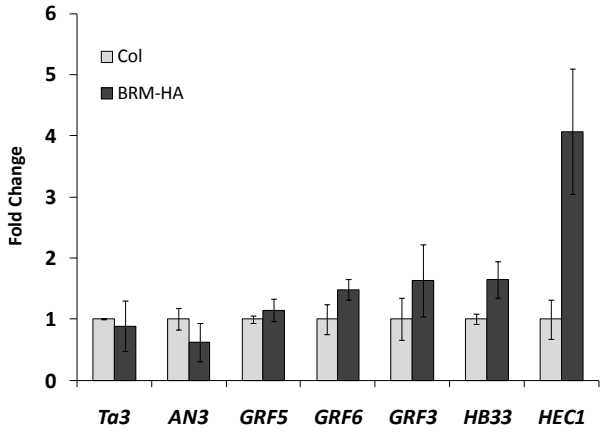


Figure 7. Chromatin immunoprecipitation shows the presence of BRM at the *HEC1* promoter.

Anti-HA antibody was used for ChIP on plants expressing HA-tagged BRM and wild-type (Col) plants, harvested at 9 DAS. qPCR was used to determine the enrichment, and threshold cycle values were normalized agianst those of wild type for each locus. *Ta3*: heterochromatic control region (Johnson et al., 2002).

Overexpression of *BRM* and *SWI3C* enhances leaf growth

Since knockout and knockdown of SWI/SNF core subunits results in severely dwarfed plants, we wondered if increased expression could lead to the development of larger organs. Only elevated SYD activity has been reported, achieved by complementation of *syd* mutants with *SYD:SYDΔC*, lacking the carboxy-terminal domain, resulting in a hypermorph phenotype opposite to the mutant phenotype (Su et al., 2006). To answer the question above, *BRM*, *SWI3C*, *SWP73B*, *ARP4* and *ARP7* were ectopically expressed from the *35S* promoter in wild-type background. For each construct, independent transformants were obtained, expression levels were quantified and individual leaf and rosette areas were measured. No consistent changes could be observed at 21 DAS for plants overexpressing *SWP73B*, *ARP4* or *ARP7* (data not shown). Increased expression of *SWI3C* on the other hand frequently led to an increase in rosette area (Fig. 8, A). Five out of thirteen *35S:SWI3C* lines had significantly larger rosette sizes (Fig. 8, A-C). Although

rosette area was similar to that of WT, closer inspection of the individual leaves of four other lines showed a strong increase in the size of the first leaves, whereas younger leaves were similar to or smaller than those of WT (Fig. 8, A and D). This phenotype is remarkably reminiscent of plants overexpressing *AN3* or *GRF5* (Fig. 4, A and B). In contrast, the remaining *35S:SWI3C* lines appeared to be smaller compared to wild-type plants, but analyses of transgene expression levels revealed some correlation with the phenotype (Supplemental Fig. 7, A). When mRNA levels were increased more than 2-fold, overexpression of *SWI3C* enhanced leaf size. In addition, pavement cell number and cell size were determined of independent *35S:SWI3C* first leaves and indicated that enhanced cell proliferation, and not cell expansion, boosts leaf growth when *SWI3C* is overexpressed (Fig. 8, E).

Likewise, the phenotype of *35S:BRM* plants could be correlated with *BRM* transcript levels, although in the opposite direction (Fig. 8, F and Supplemental Fig. 7, B). *35S:BRM* lines with increased *BRM* transcript levels compared to WT did not yield significantly enhanced leaf growth (Fig. 8, F and Supplemental Fig. 7, B and C). A more than two-fold increase in expression levels could never be obtained, suggesting that *BRM* levels are tightly controlled and most likely lethal above a certain threshold. Peculiarly, *brm*-like mutant phenotypes could be observed in some *35S:BRM* lines with increased or similar *BRM* expression compared to WT (Supplemental Fig. 7, B and C). However, shoot growth was strongly enhanced in seven out of thirteen *35S:BRM* lines, with *BRM* transcript levels that were slightly lower than wild-type levels (Fig. 8, F-J, Supplemental Fig. 7, B).

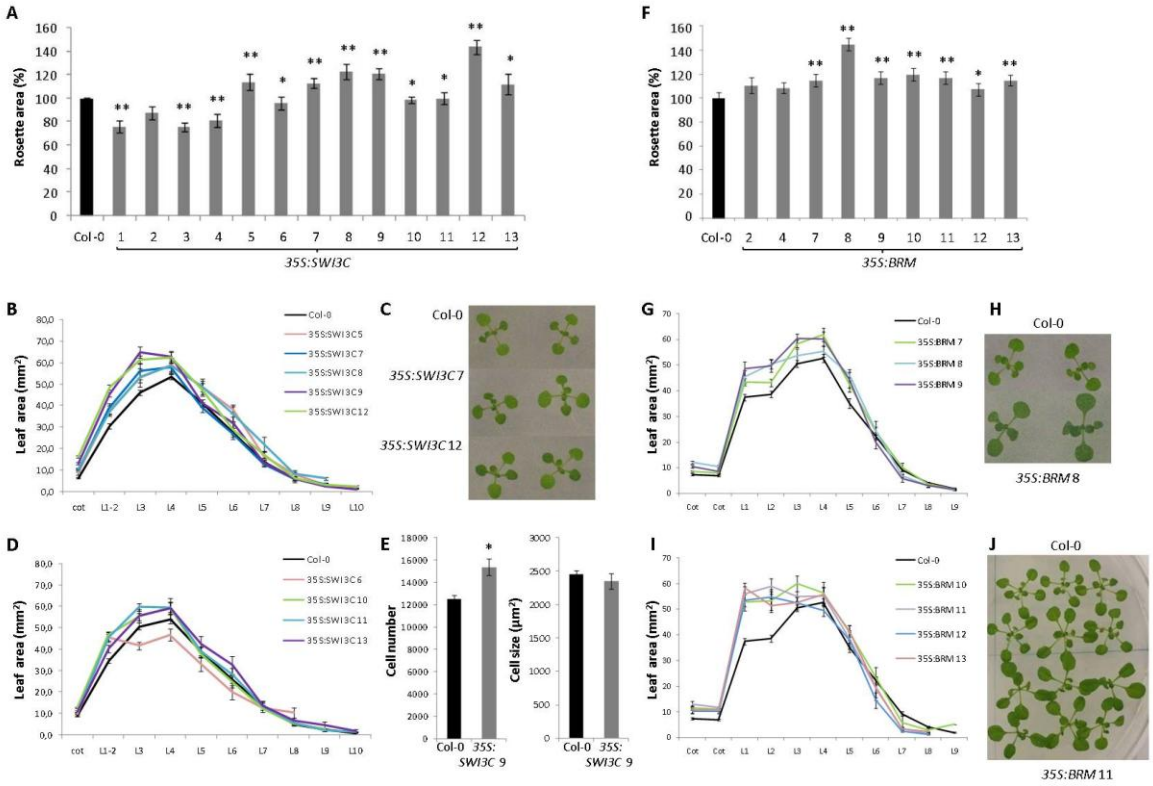


Figure 8. Modified expression of *SWI3C* and *BRM* enhances leaf growth.

A to E, *35S:SWI3C* lines. F to J, *35S:BRM* lines. A and F. Total rosette area calculated from individual leaf sizes from 21-day-old plants. Error bars are SE ($n \geq 10$). ** Significanlty different from the wild type (Col-0) ($P < 0.05$, Student's *t* test). * Rosette area is not significantly different from Col-0, but leaves 1&2 are increased in size. B, D, G and I, Individual leaf areas measured from leaf series made at 21 DAS from plants with increased leaf growth, as indicated in A and F. Cot: cotyledons. L1-L10: leaves 1 to 10. C and H, Rosettes of 15-day-old wild-type (Col-0) and representative *35S:SWI3C* or *35S:BRM* lines, showing enhanced leaf growth. E, Pavement cell number and cell area of *35S:SWI3C9*. J, Rosettes of 21-day-old wild-type (Col-0) and *35S:BRM11* plants.

DISCUSSION

By the identification of the transcriptional coactivator AN3 as an associating protein of SWI/SNF complexes in Arabidopsis, the work presented here provides an explanation for the necessity of SWI/SNF chromatin remodeling during leaf development. We hypothesize that AN3recruits, through GRFs and/or other transcription factors, BRM or SYD ATPase activity for efficient target gene transcription (Fig. 9), which acts to delay the exit from the mitotic cell cycle and simultaneously delays the start of differentiation. The microarray analysis upon AN3 activation in developing first leaves, revealing induction of genes that are downregulated and repression of genes that go up during the transition phase, respectively, support the proposed role of the SWI/SNF-AN3 complex.

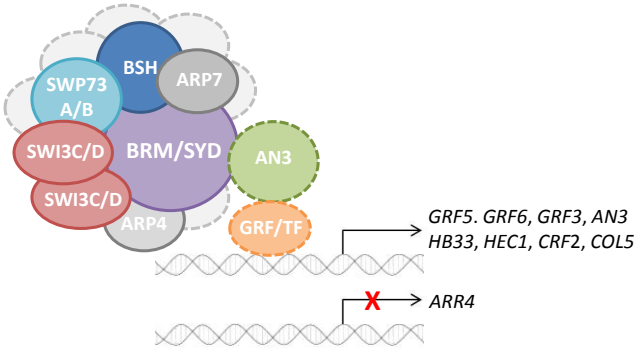


Figure 9. Model for AN3 mode of action: AN3 associates with SWI/SNF chromatin remodeling complexes for efficient target gene transcription.
AN3 is part of SWI/SNF complexes formed around a central ATPase, BRM *or* SYD, including SWP73A *or* SWP73B, SWI3C *and/or* SWI3D, ARP4 *and* ARP7, and presumably BSH and other putative subunits depicted as light grey circles. AN3 binds GRFs or putative other, yet to be identified transcription factors (TF) to recruit chromatin remodeling activity to induce or repress expression of direct and indirect target genes.

Ribosome-related processes downstream of AN3

Enhanced AN3 activity increases the duration and also the rate of cell proliferation. In order to maintain high cell proliferation rates, massive protein synthesis by ribosomes is simultaneously required (Donnelly et al., 1999; Li et al., 2005). In a growing yeast cell, >80% of total RNA and 30% to 50% of total protein was shown to be ribosomal (Warner,

1999; Perry, 2007) and during plant growth, a large portion of carbon and energy is recruited for protein synthesis and ribosome biogenesis (Piques et al., 2009). The upregulated genes after AN3 activation are enriched for genes involved in the synthesis of ribosomes, indicating that AN3 might contribute to the stimulation of ribosome biogenesis. Remarkably, ribosomal and ribosome-related proteins have been described to regulate leaf development in conjunction with *AN3* (Fujikura et al., 2009; Horiguchi et al., 2011). The combination of mutations in *an3* and *oli2* (At5g55920), most likely responsible for rRNA processing, synergistically reduces leaf cell number (Fujikura et al., 2009). Expression of *OLI2* and another closely related *OLI2*-like gene (At4g26600) was induced in *AN3-GR* leaves after DEX treatment, providing evidence for the molecular basis of the synergism between *AN3* and *OLI2*. Previous studies also showed a downregulation in *an3* of genes encoding the histone deacetylases HDT1 and HDT2 that are, like AN3, important for adaxial/abaxial leaf polarity establishment (Ueno et al., 2007; Horiguchi et al., 2011). *HDT1* and *HDT2* are induced in *AN3-GR* leaves (P-values 0.0505 and 0.0635, respectively). Moreover, HDT1 was shown to be involved in deacetylation of ribosomal DNA genes (Lawrence et al., 2004), underlining the importance of chromatin modifications and ribosome function for AN3 stimulated leaf growth.

A model for AN3/GRF action

By qRT-PCR, we identified the transcriptional regulators GRF5, GRF6, GRF3, HB33, HEC1, CRF2 and COL5 as rapidly regulated downstream targets of AN3. No other *GRFs* were differentially expressed as early as 2h after AN3 induction. *GRF5* and *GRF6* were downregulated in *an3* mutant leaves, while *GRF3* and *GRF8* were not. AN3 lacks DNA binding capacity, but has been found to interact with GRF1, GRF2, GRF4, GRF5 and GRF9 (Kim and Kende, 2004; Horiguchi et al., 2005) and based on our TAP data, GRF3 can be added to the list and associates with AN3 most likely by means of direct protein-protein binding. Taken together with the reinforcement of *AN3* transcription at 6 hours after DEX treatment, this leads to the hypothesis that AN3/GRF complexes themselves activate *AN3* and *GRF* transcription, as was reported for many transcription factors and

coactivators that bind to their own promoter. The downregulation of *AN3*, *GRF5* and *GRF6* by overexpression of *miR396* strengthens this hypothesis, since *AN3*, *GRF5* and *GRF6* do not contain a miR396-target site, in contrast to the other GRFs (Liu et al., 2009; Rodriguez et al., 2010; Wang et al., 2011a). More specifically, AN3/GRF complexes most likely activate transcription of only *GRF5*, *GRF6* and *GRF3* in proliferating leaf cells. This supports the likelihood that besides overlapping functions, GRFs have unique specialized functions that are needed for normal leaf development, corroborated by the decrease in leaf size of single *grf* mutants (Horiguchi et al., 2005; Kim and Lee, 2006) and the previously reported specific reduction in *GRF6* and *GRF9* mRNA in 10-day-old *an3* shoot tips including leaf primordia (Ichihashi et al., 2010).

Furthermore, overexpression of *GRF5* could only partially rescue the *an3* mutant phenotype and despite *an3* loss of function, *HB33* and *HEC1* expression are promoted by GRF5, while this is not the case for *CRF2* and *COL5*. Therefore, we propose a model where a complex of AN3/GRF5 regulates transcription of a subset of genes, including *HB33* and *HEC1*, while AN3 binds to other co-occurring GRFs, most likely GRF6 and GRF3, to modulate expression of other target genes like *CRF2* and *COL5*. Alternatively, AN3 can control expression of these genes independently of the GRFs by associating with other transcription factors or GRF5 can activate transcription independently of GIFs.

The presented data suggest that AN3 most likely modulates transcription by means of interaction with SWI/SNF complexes around BRM, whose remodeling activity is necessary for correct expression of *HB33*, *HEC1*, *COL5* and *CRF2*. Furthermore, *AN3* and *GRF5* expression are also dependent on functional BRM, arguing in favor of AN3/GRFs controlling their own transcription by recruiting ATPase activity. Puzzling is the correct expression of *GRF3* and *GRF6* when *brm* is mutated. However, AN3 TAP also pulled down SYD, and BRM and SYD have been shown to have overlapping and specialized functions during seedling growth, cotyledon boundary establishment, floral transition and flower formation (Farrona et al., 2004; Kwon et al., 2006; Su et al., 2006; Bezhani et al., 2007; Farrona et al., 2011; Wu et al., 2012). Further analysis should elucidate if SYD activity is needed for proper expression of specific *GRFs* when leaves develop.

A function for HB33 downstream of AN3 and BRM

HB33/ZINC FINGER HOMEODOMAIN 5 (ZHD5) is part of the zinc finger homeodomain (ZF-HD) class of transcription factors, comprising 14 members that homo- and heterodimerize and are postulated to have unique and redundant functions during Arabidopsis development (Tan and Irish, 2006; Hu et al., 2008). Overexpression of *HB33* enhances leaf growth, while overexpression of its competitive peptide inhibitor *MINI ZINC FINGER 1* (*MIF1*), that blocks nuclear import and DNA binding of HB33, results in dwarfed growth (Hu and Ma, 2006; Hong et al., 2011). These phenotypes are consistent with a positive role for HB33 downstream in the AN3/GRF5 signaling cascade. HB33, like most other ZF-HD members, lacks an intrinsic activation domain, pointing out the need for interaction with cofactors, possibly AN3, which is an interesting assumption that needs further investigation.

Although enhanced *HB33* expression is associated with AN3 activation, the gene is not repressed by *brm* mutation as expected, but induced. One explanation for these contradicting observations could be the differences in tissue sampling between both experiments. Whereas microdissected *AN3-GR* transitioning leaves were harvested after DEX induction at 8 DAS, for *brm* whole 12-day-old shoot extracts, including the SAM, were used for expression analysis. It could be that BRM functions to repress *HB33* expression in tissues that lack high AN3 levels, like expanding cells and the SAM (Horiguchi et al., 2005; Horiguchi et al., 2011; Ichihashi et al., 2011). However, *HB33* is strongly expressed in the SAM, but expression does go down in non-proliferative leaf cells (Supplemental Fig. 2, C) (Hu et al., 2008). Whole shoot extracts are enriched for differentiating leaf cells (Skirycz et al., 2010), where BRM could be recruited by other associated proteins and transcription factors to impose a repressive chromatin state on the HB33 promoter, thus resulting in a global upregulation of *HB33* expression in complete *brm* rosettes. For various SWI/SNF subunits, specific and even opposing functions have been assigned in mammals, as illustrated by the switch from self-renewal to differentiation of neurons, each promoted by SWI/SNF complexes with different compositions (Lessard et al., 2007; Wu et al., 2007; Wilson and Roberts, 2011). Furthermore, *HB33* is a direct target of the repressor AUXIN RESPONSE FACTOR 2

(ARF2), shown to have essential regulatory functions in plant growth by linking multiple hormone response pathways (Li et al., 2004; Okushima et al., 2005; Schruff et al., 2006; Vert et al., 2008; Wang et al., 2011b). ARF2 is expressed in growing leaves and whether ARF2 associates with SWI/SNF complexes containing BRM to repress *HB33*, or represses transcription by other means, provides a topic for future research.

HEC1 functions downstream of AN3 and BRM

HEC1 belongs to a small group of basic helix-loop-helix (bHLH) transcription factors with partially redundant functions in female reproductive tract development, likely by heterodimerization with another bHLH protein, SPATULA (SPT) (Gremski et al., 2007; Crawford and Yanofsky, 2011). SPT negatively regulates temperature- and/or light-sensitive cotyledon expansion and leaf growth (Ichihashi et al., 2010; Sidaway-Lee et al., 2010; Josse et al., 2011), while AN3/GRF5-mediated upregulation rather implies a positive role for HEC1 during leaf formation. Moreover, SPT was postulated to affect leaf cell proliferation independent from AN3/GRF5 (Ichihashi et al., 2010), suggesting that HEC1 does not operate together with SPT in leaf primordia, but prefers other (bHLH) transcription factors as a binding partner for its function. On the other hand, expression of *HEC1* seems to be virtually absent in wild type leaves, indicating a minor role during leaf development under our experimental conditions. *an3/gif2* mutants display defects in female floral organs (Lee et al., 2009), therefore, the possibility exists that *HEC1* is induced by AN3 to exert its effect on flower development. Alternatively, *HEC1* levels could be dependent on environmental cues, as shown for *SPT* and other bHLH proteins (Nozue et al., 2007; Sidaway-Lee et al., 2010). Extended research on *HEC* loss and gain of function mutants in different conditions will shed light on their role, if any, during leaf growth.

Although we demonstrated that *AN3*, *GRF5*, *GRF6*, *GRF3*, *HB33* and *HEC1* are transcriptional targets of AN3, that AN3 binds SWI/SNF complexes including BRM and that the expression of *AN3*, *GRF5*, *HB33* and *HEC1* depends on BRM, only the promoter of *HEC1* was physically bound by BRM. Experimental restrictions could be a putative cause of the lack of enrichment for the other promoters. Only one primer pair was tested

for each promoter, while 40% of the genomic regions bound by human BRG1 were in fact intergenic (Ho et al., 2009). Furthermore, 9-day-old whole shoot extracts were used, probably diluting the binding to loci specific for proliferating leaf cells.

A role for AN3 during the transition to flowering

COL5 shows homology to CONSTANS (CO), a major regulator of the photoperiodic flowering pathway in Arabidopsis (Putterill et al., 1995). Under inductive long days, CO accumulates in leaves due to transcriptional and post-translational stimulation by circadian clock components and light, resulting in the activation of FLOWERING LOCUS T (FT) that moves through the phloem to the SAM where it induces the transition to flowering (Imaizumi, 2010 and references therein). Like *CO*, *COL5* is expressed in the leaf vasculature, expression is under circadian control and overexpression leads to early flowering in non-inductive short day conditions concomitant with elevated *FT* levels (Hassidim et al., 2009). In our time-course experiment, we observed that *COL5* transcript levels drop during the day at 9 hours after dawn, in both DEX-treated and non-treated wild-type as well as *AN3-G*R leaves, confirming its diurnal regulation that does not seem to be affected by AN3. In addition, *COL5* expression is not going down during the different phases of leaf development (Supplemental Fig. 2, C), arguing against a specific function in leaf cell proliferation. Moreover, the microarray shows an induction of *CO* itself in *AN3-GR* leaves (P-value 0.0650), suggesting a positive role for AN3 in the photoperiodic flowering pathway by induction of *CO* and *COL5*, through BRM activity. However, several contradictory observations impede to make straightforward conclusions. First, *an3* and double/triple *gif* mutants bolt earlier in long day conditions, although rosette leaf number is not significantly different at that time due to the shorter plastochron (Lee et al., 2009). Second, *CO* expression is increased in *BRM*-silenced plants at 6 and 12 DAS (Farrona et al., 2004; Farrona et al., 2011), while our data reveal a reduction of *COL5* expression in 12-day-old *brm* shoots. Analogously, *BRM*-silenced plants flower earlier with less leaves compared to WT, while *brm* mutants flower later, but also with less leaves (Farrona et al., 2004; Hurtado et al., 2006). Furthermore, why would AN3, that functions in proliferating

cells, regulate expression of *CO* and *COL5*, whose expression domain is extended outside the division zone?

Interestingly, *ARR4* is also strongly expressed in the vasculature and the protein modulates the circadian clock redundantly with ARR3, likely in part by interaction with PHYTOCHROME B (PHYB) (Sweere et al., 2001; To et al., 2004; Salome et al., 2006; Mira-Rodado et al., 2007). Loss of ARR4 function leads to early flowering with fewer leaves, while *ARR4* overexpression delays flowering under long days (Mira-Rodado et al., 2007). Thus, the here reported repression of *ARR4* by AN3 could work in concert with the stimulation of *COL5* to promote flowering.

In addition, the homeobox transcription factor WOX13 that copurified during TAP with AN3 leads to early flowering when mutated (Deveaux et al., 2008). WOX13 is a promising candidate for interaction with AN3, although binding to other components of the SWI/SNF complex cannot be excluded. It is not surprising that several transcriptional regulators identified here, also have functions related to flowering, like AN3 and GRFs. The transformation of leaves into flowers by ectopic expression of flower homeotic and SEPALATA (SEP) genes (Honma and Goto, 2001; Pelaz et al., 2001; Ditta et al., 2004) showed that leaves and flowers are lateral organs from the same origin, with leaves being the default state.

AN3 modulates the cytokinin response pathway

The identification of CRF2 as a putative direct AN3 target gene hints at the involvement of the cytokinin response pathway downstream of AN3. Together with 11 other CRFs, CRF2 forms a subgroup within the ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (ERF) family of APETALA 2/ERF (AP2/ERF) transcription factors (Rashotte et al., 2006; Rashotte and Goertzen, 2010). Cytokinins induce *CRF2* transcription and protein translocation to the nucleus to positively regulate cytokinin-response genes, a large fraction of which are also regulated by B-type ARRs. At the same time, repression of *ARR4* by AN3 could be a general mechanism to reduce the negative feed-back inhibition on B-type *ARRs*, thereby reinforcing cytokinin signaling, known to stimulate leaf growth (Werner and Schmülling, 2009 and references therein; Holst et al., 2011).

Consistent with a role during shoot growth, triple *crf1/2/5* and *crf2/3/6* mutants display aberrant cotyledon development, characterized by a predominant reduction in cell size, deformed edges and reduced greening (Rashotte et al., 2006). Furthermore, *CRF1*, *CRF2* and *CRF3* were found to be directly repressed by PHYTOCHROME INTERACTING FACTOR 1/PHYTOCHROME INTERACTING FACTOR 3-LIKE 5 (PIF1/PIL5), which inhibits seed germination in the dark. Remarkably, *GRF5* and *HEC1* were also identified as direct targets negatively regulated by PIF1, suggesting that the same transcription factors are coordinately regulated during seed germination and seedling growth, denoting the possibility that this regulation in leaves might also depend on light.

SWI/SNF chromatin remodeling complexes recruited by AN3

Whereas the first SWI/SNF complexes have been purified from yeast and human cells more than 15 years ago (Cairns et al., 1994; Wang et al., 1996a; Wang et al., 1996b), we now succeeded in the identification of SWI/SNF complexes from cell cultures and seedlings of the plant model organism Arabidopsis. TAP with AN3, SWI3C, SWP73B, and ARP4 as baits revealed the co-occurrence of multiple complexes composed of different paralogous members of ATPase, SWI3 and SWP73 families, together with BSH, ARP4 and ARP7. Homologs for each of these proteins or protein families define yeast and human SWI/SNF complexes, underlining their evolutionary conservation (Jerzmanowski, 2007; Hargreaves and Crabtree, 2011). Because three ATPases (BRM, SYD and CHR12) were purified, at least three different complexes are present in cell culture. This number increases with the finding that SWP73A and B are mutually exclusive, but pulled down both with TAP-tagged SWI3C and ARP4, that copurified BRM as a single ATPase. As in mammals, combinatorial assembly might contribute to increase gene regulation, generating greater functional diversity. Two ARPs (yeast and human) or one ARP together with β-actin (human) are integral parts of chromatin remodeling complexes by binding specific domains of the ATPase (Zhao et al., 1998; Meagher et al., 2005; Szerlong et al., 2008; Hargreaves and Crabtree, 2011). Although actin is often considered as background and not discriminated with the TAP technique,

the isolation of two ARPs, ARP4 and ARP7, by all five baits, might indicate that, similar to yeast, actin is not part of the complexes purified here.

Solely predicted from sequence similarity before (Kim and Kende, 2004; Horiguchi et al., 2005), association of AN3 with SWI/SNF chromatin remodellers is now confirmed, both in cell cultures and seedlings. Either SYD or BRM constitute the central ATPase subunit, and based on the described interaction of the human homolog SYT with BRM and BRG1 (Thaete et al., 1999; Nagai et al., 2001; Perani et al., 2003), AN3 most likely binds BRM or SYD directly. Moreover, if stoichiometry is conserved amongst plants and mammals, the AN3 containing complexes harbor a BSH protein, SWP73A or SWP73B, ARP4 and ARP7 and two SWI3 proteins, SWI3C and/or SWI3D (Fig. 9).

Previous studies based on yeast-two-hybrid and *in vitro* pull-down experiments, identified pairwise interactions between SYD, BRM, BHS and SWI3 proteins (Fig. 5). BRM interacts with SWI3B and SWI3C, but not with SWI3A, SWI3D nor BSH (Farrona et al., 2004; Hurtado et al., 2006). BSH on the other hand, only interacts with SWI3A and SWI3B, and not with SWI3C, SWI3D or BRM (Sarnowski et al., 2002; Sarnowski et al., 2005). Consequently, it was hypothesized that core complexes around BRM have to include SWI3B, or SWI3A together with SWI3C. Furthermore, SWI3C bound SWI3A and SWI3B, SWI3D only interacted with SWI3B, while SWI3A and SWI3B were capable of homo- and heterodimerization (Sarnowski et al., 2002; Sarnowski et al., 2005). Thus, SWI3C can only form a complex with BRM and BSH if SWI3A or SWI3B are present and SWI3D can only be recruited by SWI3B (Fig. 5). However, TAP-tagged SWI3C and ARP4 only pulled down SWI3C, and AN3 TAP copurified SWI3D as well, but never SWI3A or SWI3B. Judging from the frequent identification of all SWI3 proteins with SWP73B TAP, our results are accurate and demonstrate that SWI3C can be the only SWI3-type protein in a SWI/SNF complex or can co-occur with SWI3D. If the previously described interactions are taken into account, complex assembly can be accomplished by binding of two SWI3C proteins to BRM, while BSH would have to be recruited by other subunits, e.g. SWP73A or SWP73B, or by binding of one SWI3C protein to BRM and recruitment of SWI3D and BSH by other subunits. The same composition would apply for SWI/SNF complexes around SYD and AN3, since SYD was not shown to interact with SWI3D, but with SWI3A, SWI3B and SWI3C (Fig. 5)

(Bezhani et al., 2007). Conversely, the previously used experimental systems in vitro or in yeast could not have allowed for identification of all interactions, demonstrating the need for complementary techniques *in planta*.

Furthermore, the reciprocal purification of bromodomain-containing proteins and the lack of proteins with an ARID domain, while several such proteins are present in the Arabidopsis genome (Jerzmanowski, 2007), indicate that two types of Arabidopsis SWI/SNF complexes exist, defined by similar yeast and human signature subunits with homologous domains. The complexes identified here, including the complexes that harbor AN3, resemble yeast RSC and human pBAF. This is in great contrast however to TAP of human SYT, copurifying BAF-type SWI/SNF complexes (Middeljans et al., 2012). It will be interesting to investigate if AN3 could associate with other subtype signature proteins in tissues different from those in seedlings, e.g. flowers. Bromodomains are known to bind acetylated histones (Zeng and Zhou, 2002), a function that is most likely conserved in plants, as the bromodomain of BRM was shown to bind H3 and H4 (Farrona et al., 2007). The presence of BRD proteins might be important for targeting of the remodeling activity, especially in complexes around SYD, CHR12 or CHR23 that, unlike BRM, lack an intrinsic bromodomain (Jerzmanowski, 2007). In addition, *BRD2* and *BRD5* were found to be misregulated in *brm* and *syd* mutants (Bezhani et al., 2007), strengthening the observation that the expression of genes encoding interacting proteins, like AN3, could be regulated by the SWI/SNF complex itself. The ARID domain functions to bind DNA, and although LFR shows homology with human ARID2, a typical pBAF component, the homologous protein region is situated outside the ARID domain. Nevertheless, while *lfr* mutants display pleiotropic phenotypes and *LFR* is ubiquitously expressed, the protein accumulates highly in young dividing tissues, characteristics shared with chromatin remodellers (Wang et al., 2009). Moreover, LFR resides exclusively in the nucleus where it forms nuclear speckles, reminiscent of AN3 subcellular localization (Kim and Kende, 2004), and likewise is predicted to have transcriptional activation activity without DNA binding capacity (Wang et al., 2009). Taken together, LFR could be a more general subunit of SWI/SNF complexes.

Overexpression of *SWI3C* results in increased leaf growth due to enhanced cell division and manipulation of *BRM* transcript levels also gives rise to larger rosette leaves. However, the phenotypes strongly depend on the transgene expression level, which needs to be increased above a certain threshold for *SWI3C*, but slightly decreased for *BRM* compared to WT. This can be explained by the fact that SWI/SNF complexes are of great importance to fine-tune the balance between cell proliferation and differentiation, rather than stimulating or impeding one of these two processes.

Conclusion

From the above described target gene functions, it becomes clear that AN3 activates a broad spectrum of downstream responses that allow for the integration of multiple internal and external cues, such as hormones and light, to regulate the transition from leaf cell proliferation to cell expansion and the transition from vegetative to reproductive development.

AN3 most likely associates with pBAF-type SWI/SNF complexes around BRM or SYD on the one hand, while interacting with DNA binding transcription factors on the other hand, thereby applying the ATPase activity at specific genomic regions to create more or less permissive chromatin states necessary for efficient target gene regulation during cell fate determination in the shoot.

MATERIALS AND METHODS

Cloning, construction of transgenic plants and plant material

DNA of *Arabidopsis thaliana* (L.) Heyhn. ecotype Columbia-0 (Col-0) was used to amplify the coding regions of *AN3* (At5g28640), *GRF5* (At3g13960), *BRM* (At2g46020), *SWI3C* (At1g21700), *SWP73B* (At5g14170), *ARP4* (At1g18450) and *ARP7* (At3g60830).

The *35S:AN3-GR* construct was made based on the pBI-ΔGR vector (Lloyd et al., 1994), from which the *GR* domain was amplified, flanked with attB2RAttB3 recombination sites and inserted into pDONRP2R-P3 by use of the Gateway technology (Invitrogen, http://www.invitrogen.com). *AN3* cDNA without the stop codon was flanked with AttB1AttB2 recombination sites and cloned into pDONR221, while the *CaMV35S* promoter was flanked with AttB4AttB1R sites and cloned into pDONRP4-P1R. Through Multisite Gateway cloning the fragments were introduced into pK7m34GW and pH7m34GW (Karimi et al., 2007a; Karimi et al., 2007b) resulting in the *AN3-GR* translational fusion preceded by the *35S* promoter. pK7m34GW containing *AN3-GR* was transformed into wild type Col-0 plants while *AN3-GR* in pH7m34GW was transformed in *CYCB1;1-DB-GUS* (Eloy et al., 2011) plants by floral dip, using *Agrobacterium tumefaciens* strain C58C1 pMP90.

For overexpression, *AN3*, *GRF5*, *BRM*, *SWI3C*, *SWP73B*, *ARP4* and *ARP7* cDNA clones were flanked by AttB1AttB2 recombination sites and introduced into pDONR221 and subsequently into pK7WG2 containing the *35S* promoter (Karimi et al., 2002). The *35S:AN3* construct was transformed in *grf5-1* plants, while the *35S:GRF5* construct was transformed in *an3-4* plants. Both mutants were a kind gift of Prof. Dr. Gorou Horiguchi (Horiguchi et al., 2005). *BRM*, *SWI3C*, *SWP73B*, *ARP4* and *ARP7* overexpressing constructs were transformed in wild-type Col-0 plants.

For crossing with *GRF5* overexpressing plants, kindly provided by Prof. Dr. Hirokazu Tsukaya (Horiguchi et al., 2005), an overexpression construct of *AN3* under the control of the *RolD* promoter of *Agrobacterium rhizogenes* was made by Multisite Gateway cloning. The *RolD* promoter was flanked with AttB4AttB1R sites and cloned into pDONRP4-P1R, *AN3* cDNA including stop codon was flanked with AttB1AttB2 recombination sites and cloned into pDONR221 and the *OCTOPINE SYNTHASE* (*OCS*) terminator was flanked

with attB2RAttB3 sites and inserted into pDONRP2R-P3. Subsequently, all three fragments were cloned into pKm43GW (Karimi et al., 2007a; Karimi et al., 2007b) and transformed into wild-type Col-0 plants.

Additional *35S:BRM* seeds were a kind gift of Dr. Rafal Archacki and *brm1* and *brm3* mutants were obtained from the Salk collection (http://signal.salk.edu/) and described before: *brm1* (SALK_030046) (Hurtado et al., 2006; Kwon et al., 2006) and *brm3* (SALK_088462) (Farrona et al., 2007).

Growth conditions and growth measurements

Seeds were sown *in vitro* in sterile plates containing half-strength Murashige and Skoog (½MS) medium (Murashige and Skoog, 1962) supplemented with 1% sucrose. The plates were sealed and put in a tissue culture room at 21°C under a 16-h day/8-h night regime during 21 DAS for all growth measurements. Dexamethasone (DEX), purchased from Sigma-Aldrich (D4902-1G; http://sigmaaldrich.com/), was dissolved in ethanol and added to the medium prior to sowing. To measure rosette leaf parameters, seedlings were harvested and individual leaves (cotyledons and rosette leaves) were dissected and photographed, after which leaf areas were measured with the ImageJ software (http://rsb.info.nih.gov/ij/). Rosette areas were calculated as the sum of the individual leaf areas. Cellular parameters were determined by drawings of the abaxial epidermis for five leaves with a microscope (Leica) fitted with a drawing tube and a differential interference contrast (DIC) objective. Average cell areas and corresponding leaf areas were measured with ImageJ (http://rsb.info.nih.gov/ij/), from which cell numbers were calculated.

For transcript profiling after inducible activation, the plates containing normal medium were overlaid with nylon meshes (Prosep) of 20-µm pore size to prevent roots from growing into the medium, after which seeds were sown. At 8 DAS, seedlings were transferred to plates containing mock medium or medium supplemented with DEX by gently lifting the nylon mesh with forceps. For the GUS staining, the transfer was done at 9 DAS.

GUS staining and analysis

Seedlings were harvested at 10 DAS 24h after DEX treatment and incubated in heptane for 10 min and subsequently left to dry for 5 min. Then they were submersed in 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc) buffer [100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl, 50mM NaCl buffer (pH 7.0), 2mM $K_3[Fe(CN)_6]$, and 4mM X-Gluc], vacuum infiltrated for 10 min and incubated at 37°C for 8 h. Seedlings were cleared in 100% and 70% ethanol and then kept in 90% lactic acid. Samples were mounted on slides and photographed under a stereomicroscope.

Leaf length and GUS staining were measured with the ImageJ software (http://rsb.info.nih.gov/ij/). The leaves were imaged in a horizontal position, the background was subtracted and a defined area along the length of the leaf was selected with the rectangle tool. Next, the color intensity in the rectangle was measured with a one-pixel resolution using the plot profile function. The data points were then calibrated by adjusting the distance from pixels to millimeters, and the color intensities were normalized to an arbitrary scale of 0 to 1.

ATH1 expression profiling and data analysis

Seedlings were harvested at 8 DAS 8h after DEX treatment in RNAlater solution (AM7021; AMBION), incubated at 4ºC for at least one night, after which leaves 1&2 were micro-dissected on a cold plate under a stereomicroscope and frozen in liquid nitrogen. RNA was extracted with the RNeasy mini kit with on-column DNase digestion by means of the Qiacube robot (QIAGEN). RNA samples were hybridized to single Affymetrix ATH1 Genome arrays at the VIB Nucleomics Core (Leuven, Belgium). Expression data were processed with robust multichip average (RMA) (background correction, normalization, and summarization) as implemented in BioConductor (Irizarry et al., 2003a; Irizarry et al., 2003b; Gentleman et al., 2004). Ath1121501attairgcdf_14.0.0 (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/CDF_download.asp) was used as chip definition file. The BioConductor package Limma was used to identify differentially expressed genes (Smyth, 2004). P values were corrected for multiple testing and a false discovery rate (FDR)–corrected P value < 0.05 was fused as a cutoff (Benjamini and Hochberg, 1995).

Differential transcripts were investigated with PageMan (Usadel et al., 2006) and PLAZA (Proost et al., 2009) for overrepresentation analysis. Overlap with public microarray data was calculated with Fisher exact tests (fisher.test function in R) followed by Bonferroni P value correction (Hochberg, 1988).

qRT-PCR

Complete seedlings were harvested in liquid nitrogen at 12 DAS or seedlings were harvested at 8 DAS 2, 4, and 6h after DEX treatment in RNAlater solution (AM7021; AMBION) and leaves 1&2 were microdissected under a stereomicroscope.

RNA was extracted according to a combined protocol of TRI Reagent RT (Molecular Research Center) and the RNeasy kit (QIAGEN) with on-column DNase (QIAGEN) digestion. cDNA was prepared from 500 ng to 1 μg of RNA with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. qRT-PCR was done on a LightCycler 480 (Roche Diagnostics) on 384-well plates with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's recommendations. All individual reactions were done in triplicate. Expression levels were normalized against the average of the housekeeping genes *CKA2*, and *CDKA1;1* and relative expression levels were determined with the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

nCounter analysis of mRNA expression

Seedling mRNA expression levels were measured using an nCounter Analysis System (NanoString Technologies) by the VIB Nucleomics Core (Leuven, Belgium) as described (Geiss et al., 2008). Total RNA extract (100 ng) was hybridized. Gene expression was measured simultaneously for all genes in multiplexed reactions. The nCounter code set contained probe pairs for 100 *Arabidopsis genes.* The data were normalized by a two-step procedure with internal spike-in controls and the three most stable reference genes included in the probe set (*CDKA_1*, *UBC*, and *CBP20*).

Co-immunoprecipiation (CoIP)

The *35S:AN3-HBH*, *35S:GRF5-CSFH* and *35S:GFP-CSFH* constructs were obtained by Multisite Gateway cloning in the destination vector pK7GW43D (Karimi et al., 2007a; Karimi et al., 2007b) and co-transformed in Arabidopsis cell suspension cultures. Maintenance and stable transformation of Arabidopsis PSB-D cell suspension cultures was done according to Van Leene et al. (2007).

Two-day-old PSB-D cell suspension cultures were harvested, used immediately or snap-frozen in liquid nitrogen, and stored at –70°C. Proteins were extracted after grinding in liquid nitrogen in homogenization buffer (25 mM Tris-Cl, pH 7.6, 75 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 15 mM p-nitrophenylphosphate, 60 mM β-glycerophosphate, 1 mM DTT, 0.1% Nonidet P-40, 0.1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail P9599 (Sigma-Aldrich)).

For immunoprecipitations 500 μg of total protein in homogenization buffer was incubated at 4°C for 2 h with 50 μl of 50% (v/v) anti-FlagM2 agarose (Sigma). Beads were washed three times with 500 μl homogenization buffer and used for protein gel blot analysis.

Proteins were separated by 12% SDS-PAGE and blotted onto Immobilon-P membranes (Millipore, Bedford, MA). Filters were blocked in 3% (v/v) milk powder in 25 mM Tris-Cl (pH 8), 150 mM NaCl, 0.05% Tween 20 for at least 1 h at room temperature and incubated overnight at 4°C with HA (1/1000) (Roche) or His (1/2000)(Qiagen) antibody in blocking buffer. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated IgG diluted 1/10000 (Amersham Biosciences) with a chemiluminescence system (Perkin Elmer, Norwalk, CT).

Tandem affinity purification (TAP)

A total of 9 TAP experiments were performed, including 8 from cell cultures and 1 from Arabidopsis seedlings. Amino- and carboxy-terminal fusions of AN3 to the GS tag, containing two protein G IgG-binding sites and a streptavidin-binding peptide, seperated by two tobacco etch virus cleavage sites, were expressed under the control of the *35S* promoter in Arabidopsis cell cultures. Maintenance and stable transformation of Arabidopsis PSB-D cell suspension cultures was done according to Van Leene et al. (2007). For *in planta* TAP, the fusion protein was expressed under the control of the

CDKA;1 promoter. TAP and subsequent analysis of the purified proteins by tandem matrix-assisted laser desorption ionization (MALDI) Mass Spectrometry (MS) analysis from Arabidopis cell cultures had been described in detail previously (Van Leene et al., 2011). For *in planta* TAP, Arabidopsis plants were transformed and heterozygous T3 seeds were grown in liquid medium (2.15 g/L Murashige and Skoog basal salt mixture (MS), 10 g/L sucrose, 0.5 g/L MES, 0.1 g/L myo-inositol) in shake flasks under a short day regime (8h light/16h dark). After 6 days, seedlings were harvested, frozen in N_2 (l) and stored at -80°C. Total protein extract preparation and GS TAP purification were performed as before (Van Leene et al., 2011), with some minor adaptations described in detail in Eloy et al. (2012). Purified proteins were identified by Nanoflow LC-MS/MS analyses, performed on a dual channel NanoLC Ultra 2D system (Eksigent, Dublin, California, USA) and connected to an LTQ Orbitrap Velos mass spectrometer (Thermo, Bremen, Germany), as described in Eloy et al. (2012). Spectra were processed with Proteome Discoverer 1.3.0.339 (Thermo, Bremen, Germany) and submitted for protein identification against The Arabidopsis Information Resource (TAIR) 10.0 with search engine Sequest. Proteins identified with at least 2 high confidence peptides were retained. A list of non-specific background proteins was assembled by combining our previous background list (Van Leene et al., 2011) with background proteins from control GS purifications on mock, GFP-GS, and GUS-GS cell culture extracts and GFP-GS 6-day-old plant extracts identified with Orbitrap VELOS. To obtain the final list of interactors, these background proteins were subtracted from the list of identified proteins.

Chromatin immunoprecipitation (ChIP)

Seedlings harvested at 9 DAS, transformed with HA-tagged BRM (Hurtado et al., 2006) were used for cross-linking according to a method described previously (Winter et al., 2011). Chromatin was immunoprecipitated with anti-HA antibody (Hurtado et al., 2006) and quantified by comparing the threshold cycle values between ChIP DNA and a dilution series of input DNA. The percentage of input values of the ChIP DNA was further normalized over the value obtained for the retrotransposon TA3 (Johnson et al., 2002) and presented as fold enrichment. The mean and SEM were calculated for three technical replicates and two biological replicates.

SUPPLEMENTAL DATA

Supplemental Table 1. Differentially expressed genes after induction of AN3 activity.

AGI Code	Annotation	FC	P-value	*35S:GRF5*	*an3*	WT 9-10 DAS
AT5G28640	**AN3 (ANGUSTIFOLIA 3)**	8,26	0,00031		DOWN	
AT1G35670	CDPK2 (CALCIUM-DEPENDENT PROTEIN KINASE 2)	6,03	0,00011	UP		
AT4G14680	APS3; sulfate adenylyltransferase (ATP); ATP sulfurylase	3,57	0,00146			UP
AT5G22580	unknown protein	3,49	0,03208		UP	
AT5G57660	**COL5 (CONSTANS-LIKE 5)**	3,38	0,04161		UP	
AT3G15357	unknown protein	3,15	0,00333			
AT5G67060	**HEC1 (HECATE 1)**	2,48	0,04199	UP		
AT1G51380	eukaryotic translation initiation factor 4A, putative / eIF-4A	2,46	0,03891			DOWN
AT5G56220	nucleoside-triphosphatase/ nucleotide binding	2,44	0,01975			
AT3G13960	**GRF5 (GROWTH-REGULATING FACTOR 5)**	2,32	0,01151	UP		DOWN
AT5G59130	subtilase family protein	2,26	0,04226			
AT1G64220	TOM7-2 (TRANSLOCASE OF OUTER MEMBRANE 7 KDA SUBUNIT 2)	2,04	0,00605			
AT1G62480	vacuolar calcium-binding protein-related	2,04	0,03078		DOWN	
AT5G09420	ATTOC64-V (ARABIDOPSIS THALIANA TRANSLOCON AT THE OUTER MEMBRANE OF CHLOROPLASTS 64-V)	1,99	0,04226			DOWN
AT1G05000	tyrosine specific protein phosphatase family protein	1,94	0,02776			
AT1G62510	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	1,94	0,00624		UP	
AT1G75240	**HB33 (HOMEOBOX PROTEIN 33)**	1,94	0,01975	UP		DOWN
AT2G06200	**GRF6 (GROWTH-REGULATING FACTOR 6)**	1,92	0,03288			
AT3G12340	FK506 binding / peptidyl-prolyl cis-trans isomerase	1,88	0,03288			DOWN
AT1G23100	10 kDa chaperonin, putative	1,87	0,04226			
AT5G64710	unknown protein	1,82	0,02776			
AT5G08600	U3 ribonucleoprotein (Utp) family protein	1,81	0,02776			
AT5G17270	tetratricopeptide repeat (TPR)-containing protein	1,81	0,02283			DOWN
AT4G21850	methionine sulfoxide reductase domain-containing protein / SeIR domain-containing protein	1,79	0,04161			
AT3G27550	group II intron splicing factor CRS1-related	1,77	0,03386			DOWN
AT3G26850	unknown protein	1,75	0,04199			
AT1G71850	ubiquitin thiolesterase	1,72	0,01903			
AT2G47990	SWA1 (SLOW WALKER1)	1,72	0,02169			DOWN
AT4G21880	unknown protein; Pentatricopeptide repeat	1,71	0,02955			
AT4G25730	FtsJ-like methyltransferase family protein	1,70	0,03208			DOWN
AT5G54910	DEAD/DEAH box helicase, putative	1,69	0,04865			DOWN
AT1G32190	unknown protein	1,69	0,03386	UP		DOWN
AT5G67440	NPY3 (NAKED PINS IN YUC MUTANTS 3)	1,68	0,02735			
AT1G06720	unknown protein	1,67	0,01975			DOWN
AT5G59600	pentatricopeptide (PPR) repeat-containing protein	1,66	0,04001			
AT4G26600	nucleolar protein, putative	1,65	0,04815			DOWN

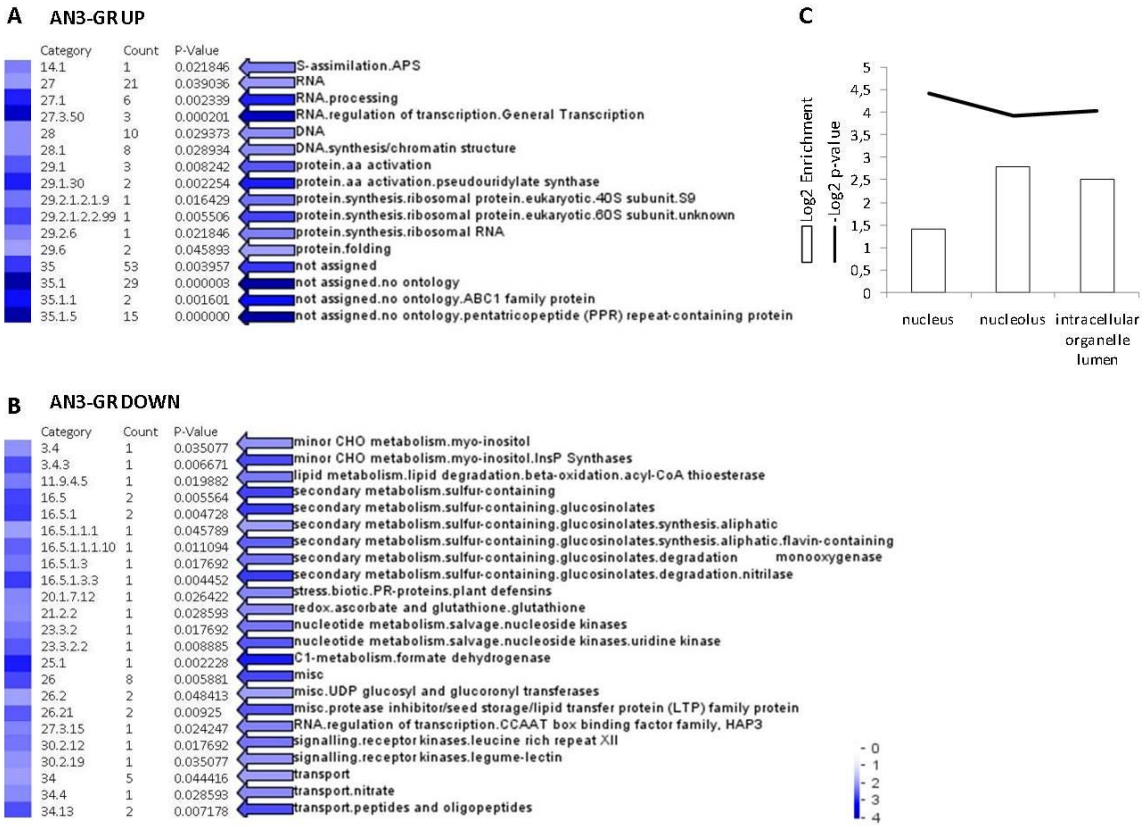
AT2G39230	pentatricopeptide (PPR) repeat-containing protein	1,64	0,03820			
AT5G60730	anion-transporting ATPase family protein	1,64	0,03386			DOWN
AT1G20370	tRNA pseudouridine synthase family protein	1,64	0,02955			
AT5G11310	pentatricopeptide (PPR) repeat-containing protein	1,64	0,03615			
AT5G27330	unknown protein	1,63	0,04001		DOWN	
AT5G07000	ST2B (SULFOTRANSFERASE 2B)	1,63	0,03386			
AT2G41990	unknown protein	1,62	0,04337			
AT5G14580	polyribonucleotide nucleotidyltransferase, putative	1,62	0,04161			DOWN
AT5G16750	TOZ (TORMOZEMBRYO DEFECTIVE)	1,61	0,02776			DOWN
AT5G59980	RNase P subunit p30 family protein	1,60	0,04161			
AT1G15870	mitochondrial glycoprotein family protein / MAM33 family protein	1,60	0,04498			DOWN
AT1G44910	protein binding	1,60	0,04289			
AT2G34570	MEE21 (maternal effect embryo arrest 21)	1,60	0,03386			DOWN
AT5G39850	40S ribosomal protein S9 (RPS9C)	1,60	0,02776			DOWN
AT1G79150	binding; unknown protein	1,60	0,03615			DOWN
AT1G14060	unknown protein	1,59	0,03891			DOWN
AT3G22660	rRNA processing protein-related	1,59	0,02776			DOWN
AT3G05130	unknown protein	1,59	0,04199			
AT5G67240	SDN3 (SMALL RNA DEGRADING NUCLEASE 3)	1,58	0,03891			
AT5G42060	unknown protein	1,58	0,04739			DOWN
AT3G13940	DNA binding / DNA-directed RNA polymerase	1,58	0,03386			DOWN
AT5G53800	unknown protein	1,58	0,04226			
AT1G61640	ABC1 family protein	1,57	0,03571			
AT3G22670	pentatricopeptide (PPR) repeat-containing protein	1,57	0,03288			
AT1G76120	tRNA pseudouridine synthase family protein	1,57	0,02955			DOWN
AT5G52630	MEF1 (MITOCHONDRIAL RNA EDITING FACTOR 1)	1,56	0,04303			
AT5G61460	MIM (hypersensitive to MMS, irradiation and MMC)	1,56	0,04865			
AT3G07050	GTP-binding family protein	1,56	0,04226		DOWN	
AT3G15080	exonuclease family protein	1,55	0,03615			DOWN
AT5G16930	AAA-type ATPase family protein	1,55	0,03066			DOWN
AT3G60980	pentatricopeptide (PPR) repeat-containing protein	1,55	0,03066			DOWN
AT4G39780	**AP2 domain-containing transcription factor, putative**	1,55	0,04592			
AT3G57150	NAP57 (Arabidopsis thaliana homologue of NAP57)	1,55	0,04536			
AT2G36400	**GRF3 (GROWTH-REGULATING FACTOR 3)**	1,54	0,04199	DOWN		DOWN
AT2G31660	SAD2 (SUPER SENSITIVE TO ABA AND DROUGHT2)	1,54	0,03386			
AT2G42870	**PAR1 (PHY RAPIDLY REGULATED 1)**	1,54	0,04161			
AT3G14900	unknown protein	1,53	0,03615			
AT5G26860	LON1 (LON PROTEASE 1)	1,53	0,04317			
AT5G06110	DNAJ heat shock N-terminal domain-containing protein / cell division protein-related	1,53	0,03386			DOWN
AT2G27360	lipase, putative	1,52	0,03615			
AT1G13160	SDA1 family protein	1,52	0,03445			DOWN
AT4G23540	binding; unknown protein	1,52	0,03347			DOWN

AT4G27380	unknown protein	1,52	0,04161		DOWN
AT3G12830	auxin-responsive family protein	1,52	0,04161		
AT4G08110	transposable element gene; CACTA-like transposase family (Ptta/En/Spm)	1,52	0,04161		
AT1G04945	unknown protein	1,52	0,04161		DOWN
AT1G10490	unknown protein	1,52	0,04317		DOWN
AT1G34160	pentatricopeptide (PPR) repeat-containing protein	1,52	0,04001		
AT4G25340	immunophilin-related / FKBP-type peptidyl-prolyl cis-trans isomerase-related	1,52	0,03386		DOWN
AT3G49320	unknown protein	1,51	0,04769		DOWN
AT1G69070	unknown protein	1,51	0,04199		DOWN
AT4G24150	**GRF8 (GROWTH-REGULATING FACTOR 8)**	1,51	0,04865		
AT3G10530	transducin family protein / WD-40 repeat family protein	1,50	0,04161		DOWN
AT1G55820	unknown protein	1,50	0,04161		
AT2G20710	pentatricopeptide (PPR) repeat-containing protein	1,50	0,03891		
AT5G11240	transducin family protein / WD-40 repeat family protein	1,50	0,04739		DOWN
AT2G40360	transducin family protein / WD-40 repeat family protein	1,50	0,04161		DOWN
AT1G49540	nucleotide binding	1,49	0,04794		
AT5G05990	mitochondrial glycoprotein family protein / MAM33 family protein	1,49	0,04289		
AT3G58660	60S ribosomal protein-related	1,49	0,04806		
AT3G59670	unknown protein	1,49	0,04001	DOWN	DOWN
AT2G21440	RNA recognition motif (RRM)-containing protein	1,48	0,03386		
AT2G41820	leucine-rich repeat transmembrane protein kinase, putative	1,48	0,04001		
AT5G61400	pentatricopeptide (PPR) repeat-containing protein	1,47	0,04815		
AT3G48250	pentatricopeptide (PPR) repeat-containing protein	1,47	0,04739		
AT1G11390	ABC1 family protein	1,47	0,04161		
AT2G47250	RNA helicase, putative	1,47	0,04161		
AT4G12740	adenine-DNA glycosylase-related / MYH-related	1,46	0,04632		
AT4G23750	**CRF2 (CYTOKININ RESPONSE FACTOR 2)**	1,46	0,04161		
AT1G15480	DNA binding; unknown protein	1,45	0,04001		DOWN
AT3G25970	pentatricopeptide (PPR) repeat-containing protein	1,45	0,04937		
AT5G60040	NRPC1	1,45	0,04815		
AT5G46680	pentatricopeptide (PPR) repeat-containing protein	1,44	0,04865		
AT2G40430	unknown protein	1,44	0,04289		DOWN
AT1G13410	unknown protein	1,43	0,04199		
AT1G63810	unknown protein	1,43	0,04815		DOWN
AT5G42450	pentatricopeptide (PPR) repeat-containing protein	1,43	0,04937		
AT4G26110	NAP1;1 (NUCLEOSOME ASSEMBLY PROTEIN1;1)	1,43	0,04199		
AT2G37400	chloroplast lumen common family protein	1,42	0,04265		DOWN
AT5G56090	COX15 (cytochrome c oxidase 15)	1,42	0,04592		
AT5G06550	unknown protein	1,42	0,04317		DOWN
AT5G55920	nucleolar protein, putative	1,41	0,04436		DOWN
AT3G27730	RCK (ROCK-N-ROLLERS)	1,41	0,04865		

AT5G24240	phosphatidylinositol 3- and 4-kinase family protein / ubiquitin family protein	-5,85	0,00011	DOWN			
AT3G30720	QQS (QUA-QUINE STARCH)	-3,91	0,00337	DOWN			
AT1G26190	phosphoribulokinase/uridine kinase family protein	-3,82	0,00011				
AT2G01890	PAP8 (PURPLE ACID PHOSPHATASE 8)	-2,65	0,03206		UP		
AT5G55450	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	-2,44	0,00309				UP
AT1G60590	polygalacturonase, putative / pectinase, putative	-2,24	0,01975				UP
AT5G22300	NIT4 (NITRILASE 4)	-2,13	0,04632	DOWN			
AT4G39800	MIPS1 (MYO-INOSITOL-1-PHOSTPATE SYNTHASE 1)	-2,05	0,02169				UP
AT5G48485	DIR1 (DEFECTIVE IN INDUCED RESISTANCE 1)	-2,03	0,03103				
AT3G16120	dynein light chain, putative	-2,00	0,03347				
AT1G65490	unknown protein	-1,99	0,01151				UP
AT2G41090	calmodulin-like calcium-binding protein, 22 kDa (CaBP-22)	-1,91	0,03445	DOWN			UP
AT2G43520	ATTI2; serine-type endopeptidase inhibitor	-1,85	0,01375	UP			
AT1G03870	FLA9 (FASCICLIN-LIKE ARABINOOGALACTAN 9)	-1,81	0,02776		UP		
AT1G62560	FMO GS-OX3 (FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 3)	-1,80	0,04199				
AT3G55710	UDP-glucoronosyl/UDP-glucosyl transferase	-1,78	0,03075				UP
AT3G21870	CYCP2;1 (cyclin p2;1)	-1,77	0,01903				
AT4G02410	lectin protein kinase family protein	-1,75	0,04226				
AT3G50440	MES10 (METHYL ESTERASE 10)	-1,74	0,03288		UP		
AT1G69870	proton-dependent oligopeptide transport (POT) family protein	-1,73	0,04337		UP		
AT1G78070	unknown protein	-1,70	0,03326				
AT5G24530	DMR6 (DOWNY MILDEW RESISTANT 6)	-1,69	0,03288	DOWN	UP		UP
AT4G10310	HKT1 (HIGH-AFFINITY K+ TRANSPORTER 1)	-1,69	0,04161				
AT3G11410	PP2CA (ARABIDOPSIS THALIANA PROTEIN PHOSPHATASE 2CA)	-1,66	0,03615				
AT1G68570	proton-dependent oligopeptide transport (POT) family protein	-1,65	0,02735	DOWN			UP
AT1G51700	**ADOF1 (Dof zinc finger protein)**	-1,65	0,04161				
AT2G26690	nitrate transporter (NTP2)	-1,64	0,04001		UP		
AT5G14780	FDH (FORMATE DEHYDROGENASE)	-1,64	0,04199		UP		
AT1G71030	**MYBL2 (MYB-LIKE 2)**	-1,64	0,03820		UP		
AT5G19730	pectinesterase family protein	-1,61	0,03386				
AT1G26560	BGLU40 (BETA GLUCOSIDASE 40)	-1,60	0,03208		DOWN		
AT2G04039	unknown protein	-1,59	0,03386				UP
AT2G03550	hydrolase	-1,59	0,04001				
AT4G22200	AKT2/3 (ARABIDOPSIS POTASSIUM TRANSPORT 2/3)	-1,56	0,04161				UP
AT1G15740	leucine-rich repeat family protein	-1,56	0,04001				
AT4G33660	unknown protein	-1,56	0,02776				
AT4G08850	kinase	-1,55	0,04161				
AT5G66170	unknown protein	-1,55	0,03288				
AT4G09900	MES12 (METHYL ESTERASE 12)	-1,52	0,03288				
AT5G47640	**NF-YB2 (NUCLEAR FACTOR Y, SUBUNIT B2)**	-1,52	0,03288				
AT1G03610	unknown protein	-1,49	0,04739				

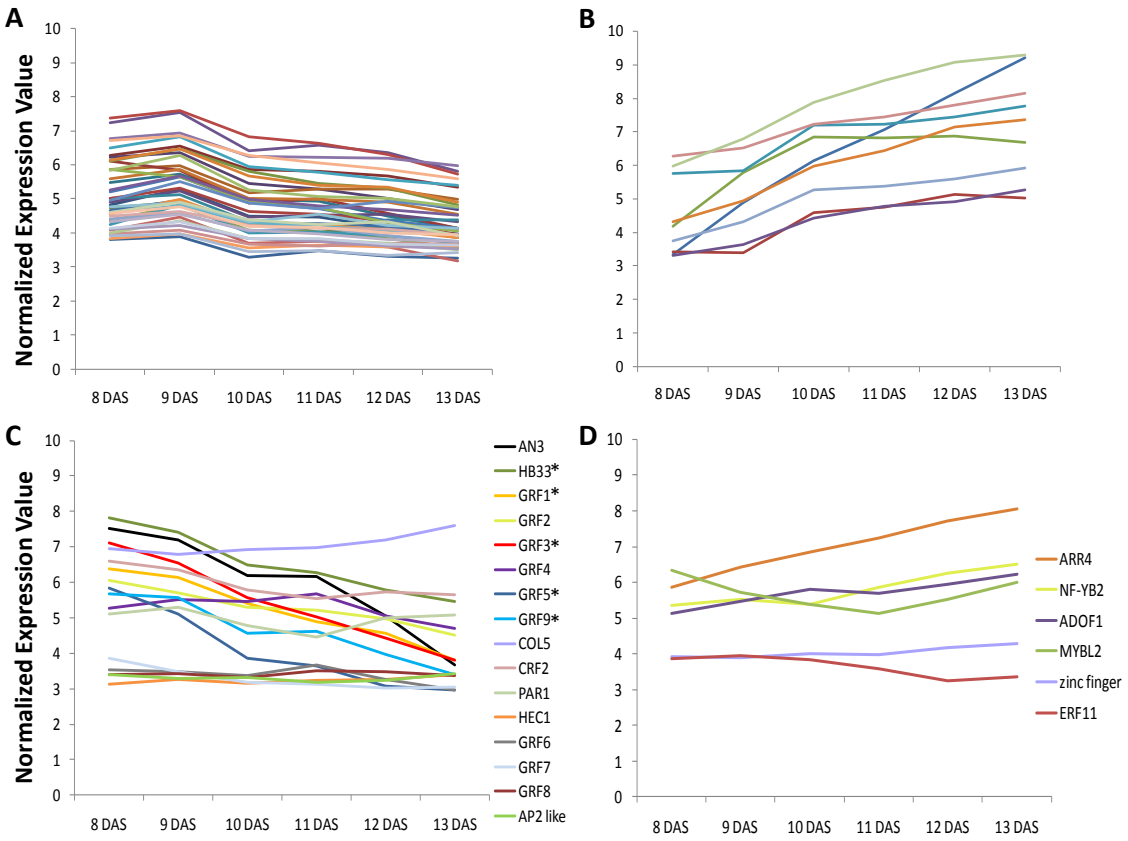
AT3G22104	phototropic-responsive NPH3 protein-related	-1,49	0,04001		
AT1G28370	**ERF11 (ERF DOMAIN PROTEIN 11)**	-1,48	0,04085		
AT5G43740	disease resistance protein (CC-NBS-LRR class), putative	-1,44	0,04226		
AT3G29240	unknown protein	-1,43	0,04739		UP
AT5G66070	**zinc finger (C3HC4-type RING finger) family protein**	-1,43	0,04536		
AT2G31570	ATGPX2 (GLUTATHIONE PEROXIDASE 2)	-1,42	0,04226		
AT3G21750	UGT71B1 (UDP-GLUCOSYL TRANSFERASE 71B1)	-1,39	0,04865		

Genes differentially expressed with P-value < 0.05 in *AN3-GR* leaves 1&2 compared to Col-0 leaves 8h after DEX treatment as determined by Affymetrix ATH1 microarrays. Transcription factors are indicated in bold. Upregulated (no shading) and downregulated (grey shading) genes are ordered according to Fold Change (FC). Columns 5, 6 and 7 indicate the intersection with publicly available microarray datasets of *35S:GRF5* shoots (Gonzalez et al., 2010), *an3* leaves 1&2 (Horiguchi et al., 2011) and wild-type (WT) Col-0 leaf 3 between days 9 and 10 (Andriankaja et al., 2012) (see Fig. 3). DOWN: downregulated. UP: upregulated.



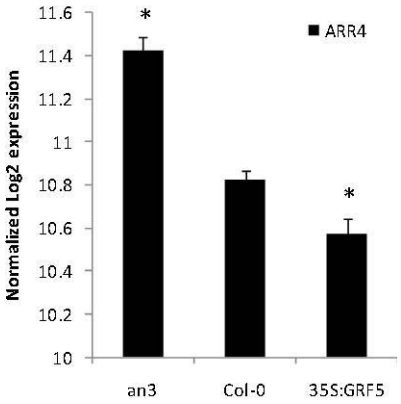
Supplemental Figure 1. Overrepresentation analysis of differentially expressed genes after AN3 induction.

A and B, PageMan (Usadel et al., 2006) enrichments of functional categories amongst genes upregulated in *AN3-GR* (A) and genes downregulated in *AN3-GR* (B). C, PLAZA (Proost et al., 2009) enrichment of subcellular localization for upregulated genes in *AN3-GR*.



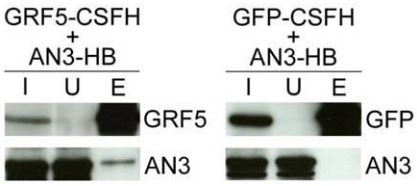
Supplemental Figure 2. Expression profiles of selected genes during leaf 3 development from proliferation to expansion.

Normalized expression values were calculated from AGRONOMICS1 tiling arrays by Andriankaja et al. (2012). A, Genes from the intersection between *AN3-GR* UP and WT 9-10 DAS DOWN, as shown in Fig. 2, A. B, Genes from the intersection between *AN3-GR* DOWN and WT 9-10 DAS UP, as shown in Fig. 2, B. C, Transcription factors upregulated in *AN3-GR*, including *AN3* and the remaining *GRF*s. D, Transcription factors downregulated in *AN3-GR*. * Significantly differentially expressed between days 9 and 10 (P-value < 0.05).



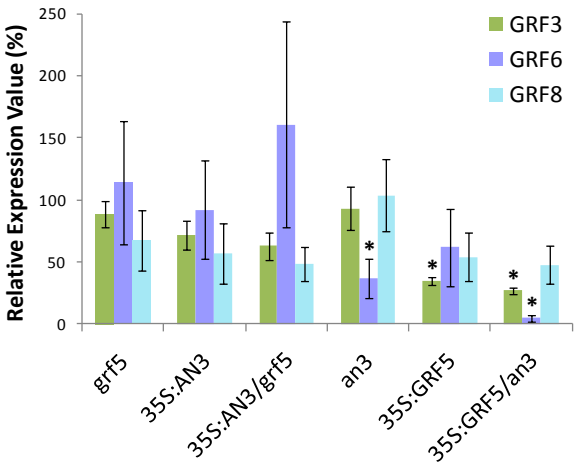
Supplemental Figure 3. Normalized ARR4 expression.

Transcript levels were determined in 6-day-old shoots, or microdissected leaves 1&2 by the nCounter nanostring technology. Error bars are SE ($n \geq 3$). * Significantly different from Col-0 plants ($P < 0.01$, Student's *t* test).



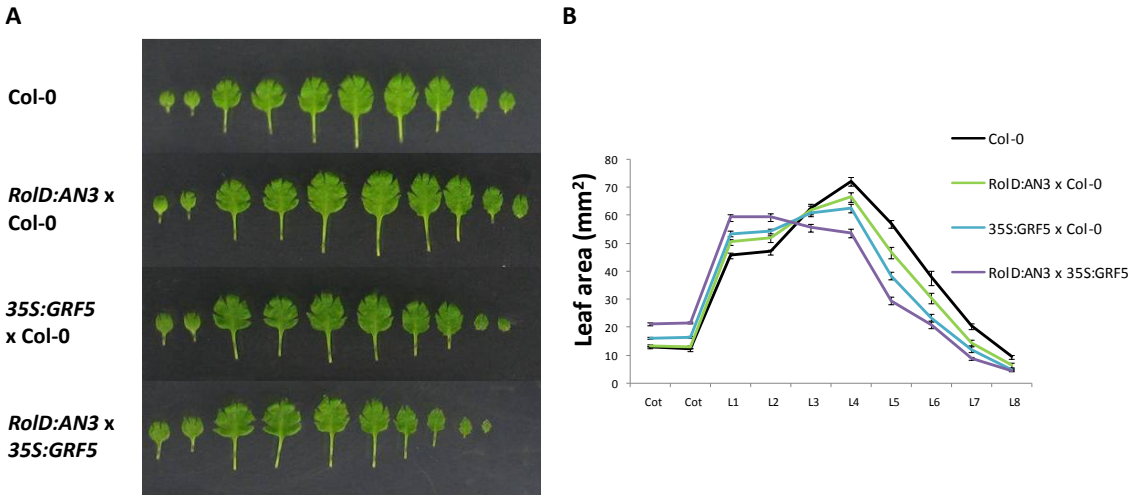
Supplemental Figure 4. Co-immunoprecipitation of AN3 and GRF5.

Immunoprecipitated GRF5-CSFH or GFP-CSFH complexes from total protein extracts of *35S:GRF5-CSFH* plus *35S:AN3-HBH* and *35S:GFP-CSFH* plus *35S:AN3-HBH* co-transformed cell suspension cultures were analyzed by immunoblot analysis with anti-HA (upper lane), and anti-His (lowest panel) antibodies. $1/10^{th}$ of the total input protein extract (I), $1/10^{th}$ of the unbound (U) fraction and the total bead bound fraction (E) were loaded.



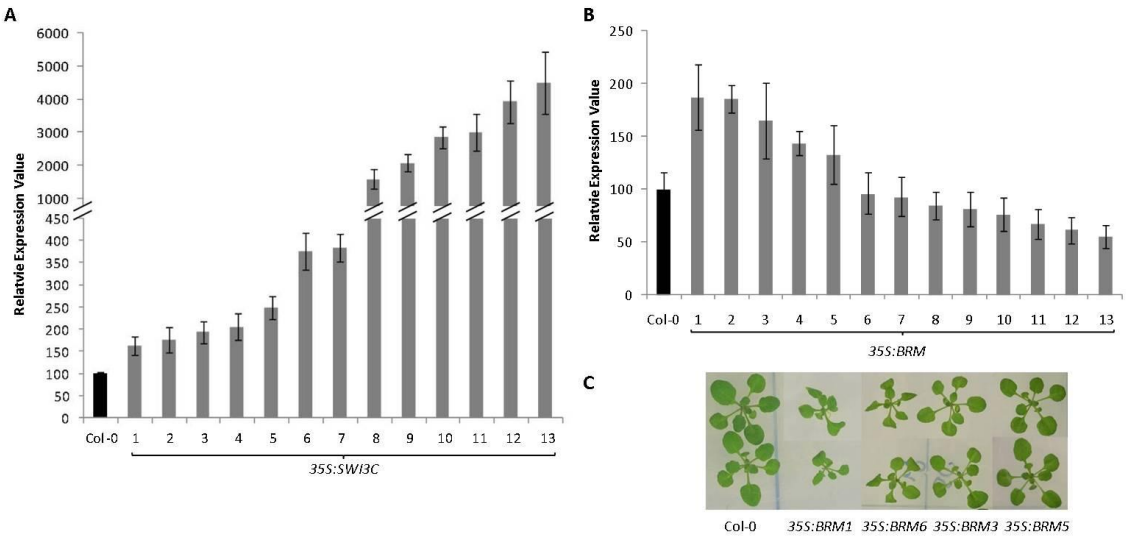
Supplemental Figure 5. *GRF* expression in transgenic lines with altered *GRF5* and/or *AN3* transcript levels.

Relative *GRF3*, *GRF6* and *GRF8* expression levels in 12-day-old shoots of transgenic lines, for each gene normalized to wild-type (Col-0) expression level which is set to 100%. Error bars are SE (n = 3). * Significantly different from Col-0 plants ($P < 0.075$, Student's *t* test).



Supplemental Figure 6. Increased leaf growth resulting from combined *AN3* and *GRF5* overexpression.

A, Leaf series of 21-day-old wild-type (Col-0), *RolD:AN3* crossed with *35S:GRF5*, and heterozygous control plants. B, Area of cotyledons (Cot) and leaves 1 to 8 (L1-L8), measured from leaf series of 21-day-old plants. Error bars indicate SE ($n \geq 14$).



Supplemental Figure 7. *35S:SWI3C* and *35S:BRM*.

A and B, Relative expression levels of *SWI3C* (A) and *BRM* (B) in different independent transgenic lines, determined by qRT-PCR and normailized to wild-type (Col-0) expression levels. Error bars are SE ($n \geq 2$). C, Rosettes of 21-day-old wild-type (Col-0) and *35S:BRM* lines, showing a *brm*-like mutant phenotype (*35S:BRM1* and *35S:BRM6*) or no phenotype (*35S:BRM3* and *35S:BRM5*).

REFERENCES

Andriankaja M, Dhondt S, De Bodt S, Vanhaeren H, Coppens F, De Milde L, Mühlenbock P, Skirycz A, Gonzalez N, Beemster GTS, Inzé D (2012) Exit from proliferation during leaf development in *Arabidopsis thaliana*: a not-so-gradual process. Developmental Cell **22:** 64-78

Archacki R, Sarnowski TJ, Halibart-Puzio J, Brzeska K, Buszewicz D, Prymakowska-Bosak M, Koncz C, Jerzmanowski A (2009) Genetic analysis of functional redundancy of BRM ATPase and ATSWI3C subunits of Arabidopsis SWI/SNF chromatin remodelling complexes. Planta **229:** 1281-1292

Beemster GTS, De Veylder L, Vercruysse S, West G, Rombaut D, Van Hummelen P, Galichet A, Gruissem W, Inzé D, Vuylsteke M (2005) Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. Plant Physiology **138:** 734-743

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate - a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B, Statistical Methodology **57:** 289-300

Bezhani S, Winter C, Hershman S, Wagner JD, Kennedy JF, Kwon CS, Pfluger J, Su YH, Wagner D (2007) Unique, shared, and redundant roles for the Arabidopsis SWI/SNF chromatin remodeling ATPases BRAHMA and SPLAYED. Plant Cell **19:** 403-416

Brzeski J, Podstolski W, Olczak K, Jerzmanowski A (1999) Identification and analysis of the Arabidopsis thaliana BSH gene, a member of the SNF5 gene family. Nucleic Acids Research **27:** 2393-2399

Cairns BR, Kim YJ, Sayre MH, Laurent BC, Kornberg RD (1994) A Multisubunit Complex Containing the Swi1/Adr6, Swi2/Snf2, Swi3, Snf5, and Snf6 Gene-Products Isolated from Yeast. Proceedings of the National Academy of Sciences of the United States of America **91:** 1950-1954

Colón-Carmona A, You R, Haimovitch-Gal T, Doerner P (1999) Spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. Plant Journal **20:** 503-508

Crane YM, Gelvin SB (2007) RNAi-mediated gene silencing reveals involvement of Arabidopsis chromatin-related genes in Agrobacterium-mediated root transformation. Proceedings of the National Academy of Sciences of the United States of America **104:** 15156-15161

Crawford BCW, Yanofsky MF (2011) HALF FILLED promotes reproductive tract development and fertilization efficiency in Arabidopsis thaliana. Development **138:** 2999-3009

Deveaux Y, Toffano-Nioche C, Claisse G, Thareau V, Morin H, Laufs P, Moreau H, Kreis M, Lecharny A (2008) Genes of the most conserved WOX clade in plants affect root and flower development in Arabidopsis. Bmc Evolutionary Biology **8**

Ditta G, Pinyopich A, Robles P, Pelaz S, Yanofsky MF (2004) The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Current Biology **14:** 1935-1940

Donnelly PM, Bonetta D, Tsukaya H, Dengler RE, Dengler NG (1999) Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. Developmental Biology **215:** 407-419

Eloy NB, de Freitas Lima M, Van Damme D, Vanhaeren H, Gonzalez N, De Milde L, Hemerly AS, Beemster GTS, Inzé D, Ferreira PCG (2011) The APC/C *subunit 10* plays an essential role in cell proliferation during leaf development. Plant Journal **68:** 351-363

Eloy NB, Gonzalez N, Van Leene J, Maleux K, Vanhaeren H, De Milde L, Dhondt S, Vercruysse L, Witters E, Mercier R, Cromer L, Beemster GT, Remaut H, Van Montagu MCE, De Jaeger G, Ferreira PCG, Inzé D (2012) SAMBA, a plant-specific anaphase-promoting complex/cyclosome regulator is involved in early development and A-type cyclin stabilization. Proc Natl Acad Sci U S A E-pub ahead of print

Eshed Y, Izhaki A, Baum SF, Floyd SK, Bowman JL (2004) Asymmetric leaf development and blade expansion in Arabidopsis are mediated by KANADI and YABBY activities. Development **131:** 2997-3006

Farrona S, Hurtado L, Bowman JL, Reyes JC (2004) The Arabidopsis thaliana SNF2 homolog AtBRM controls shoot development and flowering. Development **131:** 4965-4975

Farrona S, Hurtado L, March-Diaz R, Schmitz RJ, Florencio FJ, Turck F, Amasino RM, Reyes JC (2011) Brahma Is Required for Proper Expression of the Floral Repressor FLC in Arabidopsis. Plos One **6**

Farrona S, Hurtado L, Reyes JC (2007) A nucleosome interaction module is required for normal function of Arabidopsis thaliana BRAHMA. Journal of Molecular Biology **373:** 240-250

Fatica A, Tollervey D (2002) Making ribosomes. Current Opinion in Cell Biology **14:** 313-318

Fujikura U, Horiguchi G, Ponce MR, Micol JL, Tsukaya H (2009) Coordination of cell proliferation and cell expansion mediated by ribosome-related processes in the leaves of Arabidopsis thaliana. Plant Journal **59:** 499-508

Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, James JJ, Maysuria M, Mitton JD, Oliveri P, Osborn JL, Peng T, Ratcliffe AL, Webster PJ, Davidson EH, Hood L, Dimitrov K (2008) Direct multiplexed measurement of gene expression with color-coded probe pairs. Nature Biotechnology **26:** 317-325

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JYH, Zhang J (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biology **5:** R80

Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhondt S, Van Daele T, De Milde L, Weigel D, Kamiya Y, Stitt M, Beemster GTS, Inzé D (2010) Increased leaf size: different means to an end. Plant Physiology **153:** 1261-1279

Gonzalez N, Vanhaeren H, Inzé D (2012) Leaf size control: complex coordination of cell division and expansion. Trends in Plant Science **in press**

Gremski K, Ditta G, Yanofsky MF (2007) The HECATE genes regulate female reproductive tract development in Arabidopsis thaliana. Development **134:** 3593-3601

Han P, Li Q, Zhu YX (2008) Mutation of Arabidopsis BARD1 causes meristem defects by failing to confine WUSCHEL expression to the organizing center. Plant Cell **20:** 1482-1493

Hargreaves DC, Crabtree GR (2011) ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. Cell Research **21:** 396-420

Hassidim M, Harir Y, Yakir E, Kron I, Green RM (2009) Over-expression of CONSTANS-LIKE 5 can induce flowering in short-day grown Arabidopsis. Planta **230:** 481-491

Ho LN, Jothi R, Ronan JL, Cui KR, Zhao KJ, Crabtree GR (2009) An embryonic stem cell chromatin remodeling complex, esBAF, is an essential component of the core pluripotency transcriptional network. Proceedings of the National Academy of Sciences of the United States of America **106:** 5187-5191

Hochberg Y (1988) A Sharper Bonferroni Procedure for Multiple Tests of Significance. Biometrika **75:** 800-802

Holst K, Schmulling T, Werner T (2011) Enhanced cytokinin degradation in leaf primordia of transgenic Arabidopsis plants reduces leaf size and shoot organ primordia formation. Journal of Plant Physiology **168:** 1328-1334

Hong S-Y, Kim O-K, Kim S-G, Yang M-S, Park C-M (2011) Nuclear import and DNA binding of the ZHD5 transcription factor is modulated by a competitive peptide inhibitor in *Arabidopsis*. Journal of Biological Chemistry **286:** 1659-1668

Honma T, Goto K (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature **409:** 525-529

Horiguchi G, Kim G-T, Tsukaya H (2005) The transcription factor AtGRF5 and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of *Arabidopsis thaliana*. Plant Journal **43:** 68-78

Horiguchi G, Nakayama H, Ishikawa N, Kubo M, Demura T, Fukuda H, Tsukaya H (2011) ANGUSTIFOLIA3 Plays Roles in Adaxial/Abaxial Patterning and Growth in Leaf Morphogenesis. Plant and Cell Physiology **52:** 112-124

Hu W, dePamphilis CW, Ma H (2008) Phylogenetic analysis of the plant-specific Zinc Finger-Homeobox and Mini Zinc Finger gene families. Journal of Integrative Plant Biology **50:** 1031-1045

Hu W, Ma H (2006) Characterization of a novel putative zinc finger gene MIF1: involvement in multiple hormonal regulation of Arabidopsis development. Plant Journal **45:** 399-422

Hurtado L, Farrona S, Reyes JC (2006) The putative SWI/SNF complex subunit BRAHMA activates flower homeotic genes in Arabidopsis thaliana. Plant Mol Biol **62:** 291-304

Ichihashi Y, Horiguchi G, Gleissberg S, Tsukaya H (2010) The bHLH Transcription Factor SPATULA Controls Final Leaf Size in Arabidopsis thaliana. Plant and Cell Physiology **51:** 252-261

Ichihashi Y, Kawade K, Usami T, Horiguchi G, Takahashi T, Tsukaya H (2011) Key Proliferative Activity in the Junction between the Leaf Blade and Leaf Petiole of Arabidopsis. Plant Physiol **157:** 1151-1162

Imaizumi T (2010) Arabidopsis circadian clock and photoperiodism: time to think about location. Current Opinion in Plant Biology **13:** 83-89

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003a) Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Research **31:** e15

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003b) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:** 249-264

Jerzmanowski A (2007) SWI/SNF chromatin remodeling and linker histones in plants. Biochimica Et Biophysica Acta-Gene Structure and Expression **1769:** 330-345

Johnson LM, Cao XF, Jacobsen SE (2002) Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. Current Biology **12:** 1360-1367

Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant MicroRNAs and their targets, including a stress-induced miRNA. Molecular Cell **14:** 787-799

Josse E-M, Gan Y, Bou-Torrent J, Stewart KL, Gilday AD, Jeffree CE, Vaistij FE, Martínez-García JF, Nagy F, Graham IA, Halliday KJ (2011) A DELLA in disguise: SPATULA restrains the growth of the developing *Arabidopsis* seedling. Plant Cell **23:** 1337-1351

Kandasamy MK, Deal RB, McKinney EC, Meagher RB (2005a) Silencing the nuclear actin-related protein AtARP4 in Arabidopsis has multiple effects on plant development, including early flowering and delayed floral senescence. Plant Journal **41:** 845-858

Kandasamy MK, McKinney EC, Deal RB, Meagher RB (2005b) Arabidopsis ARP7 is an essential actin-related protein required for normal embryogenesis, plant architecture, and floral organ abscission. Plant Physiol **138:** 2019-2032

Karimi M, Bleys A, Vanderhaeghen R, Hilson P (2007a) Building blocks for plant gene assembly. Plant Physiology **145:** 1183-1191

Karimi M, Depicker A, Hilson P (2007b) Recombinational cloning with plant gateway vectors. Plant Physiology **145:** 1144-1154

Karimi M, Inzé D, Depicker A (2002) GATEWAY^{TM} vectors for *Agrobacterium*-mediated plant transformation. Trends in Plant Science **7:** 193-195

Kazama T, Ichihashi Y, Murata S, Tsukaya H (2010) The mechanism of cell cycle arrest front progression explained by a *KLUH/CYP78A5*-dependent mobile growth factor in developing leaves of *Arabidopsis thaliana*. Plant and Cell Physiology **51:** 1046-1054

Kim JH, Choi D, Kende H (2003) The AtGRF family of putative transcription factors is involved in leaf and cotyledon growth in *Arabidopsis*. Plant Journal **36:** 94-104

Kim JH, Kende H (2004) A transcriptional coactivator, AtGIF1, is involved in regulating leaf growth and morphology in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **101:** 13374-13379

Kim JH, Lee BH (2006) GROWTH-REGULATING FACTOR4 of Arabidopsis thaliana is required for development of leaves, cotyledons, and shoot apical meristem. Journal of Plant Biology **49:** 463-468

Kwon CS, Chen C, Wagner D (2005) *WUSCHEL* is a primary target for transcriptional regulation by SPLAYED in dynamic control of stem cell fate in *Arabidopsis*. Genes & Development **19:** 992-1003

Kwon CS, Hibara K, Pfluger J, Bezhani S, Metha H, Aida M, Tasaka M, Wagner D (2006) A role for chromatin remodeling in regulation of CUC gene expression in the Arabidopsis cotyledon boundary. Development **133:** 3223-3230

Kwon CS, Wagner D (2007) Unwinding chromatin for development and growth: a few genes at a time. Trends in Genetics **23:** 403-412

Lawrence RJ, Earley K, Pontes O, Silva M, Chen ZJ, Neves N, Viegas W, Pikaard CS (2004) A concerted DNA methylation/histone methylation switch regulates rRNA gene dosage control and nucleolar dominance. Molecular Cell **13:** 599-609

Lee BH, Ko J-H, Lee S, Lee Y, Pak J-H, Kim JH (2009) The Arabidopsis *GRF-INTERACTING FACTOR* gene family performs an overlapping function in determining organ size as well as multiple developmental properties. Plant Physiology **151:** 655-668

Lessard J, Wu JI, Ranish JA, Wan M, Winslow MM, Staahl BT, Wu H, Aebersold R, Graef IA, Crabtree GR (2007) An essential switch in subunit composition of a chromatin remodeling complex during neural development. Neuron **55:** 201-215

Li CX, Potuschak T, Colon-Carmona A, Gutierrez RA, Doerner P (2005) Arabidopsis TCP20 links regulation of growth and cell division control pathways. Proceedings of the National Academy of Sciences of the United States of America **102:** 12978-12983

Li H, Johnson P, Stepanova A, Alonso JM, Ecker JR (2004) Convergence of signaling pathways in the control of differential cell growth in *Arabidopsis*. Developmental Cell **7:** 193-204

Liu D, Song Y, Chen Z, Yu D (2009) Ectopic expression of miR396 suppresses *GRF* target gene expression and alters leaf growth in *Arabidopsis*. Physiologia Plantarum **136:** 223-236

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:** 402-408

Lloyd AM, Schena M, Walbot V, Davis RW (1994) Epidermal-Cell Fate Determination in Arabidopsis - Patterns Defined by a Steroid-Inducible Regulator. Science **266:** 436-439

Maston GA, Evans SK, Green MR (2006) Transcriptional regulatory elements in the human genome. Annual Review of Genomics and Human Genetics **7:** 29-59

Meagher RB, Deal RB, Kandasamy MK, McKinney EC (2005) Nuclear actin-related proteins as epigenetic regulators of development. Plant Physiol **139:** 1576-1585

Middeljans E, Wan X, Jansen PW, Sharma V, Stunnenberg HG, Logie C (2012) SS18 together with animal-specific factors defines human BAF-type SWI/SNF complexes. Plos One **7:** e33834

Mira-Rodado V, Sweere U, Grefen C, Kunkel T, Fejes E, Nagy F, Schafer E, Harter K (2007) Functional cross-talk between two-component and phytochrome B signal transduction in Arabidopsis. J Exp Bot **58:** 2595-2607

Mohrmann L, Verrijzer CP (2005) Composition and functional specificity of SWI2/SNF2 class chromatin remodeling complexes. Biochimica Et Biophysica Acta-Gene Structure and Expression **1681:** 59-73

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum **15:** 473-497

Nagai M, Tanaka S, Tsuda M, Endo S, Kato H, Sonobe H, Minami A, Hiraga H, Nishihara H, Sawa H, Nagashima K (2001) Analysis of transforming activity of human synovial sarcoma-associated chimeric protein SYT-SSX1 bound to chromatin remodeling factor hBRM/hSNF2 alpha. Proceedings of the National Academy of Sciences of the United States of America **98:** 3843-3848

Nozue K, Covington MF, Duek PD, Lorrain S, Fankhauser C, Harmer SL, Maloof JN (2007) Rhythmic growth explained by coincidence between internal and external cues. Nature **448:** 358-U311

Okushima Y, Mitina I, Quach HL, Theologis A (2005) AUXIN RESPONSE FACTOR 2 (ARF2): a pleiotropic developmental regulator. Plant Journal **43:** 29-46

Pandey R, Muller A, Napoli CA, Selinger DA, Pikaard CS, Richards EJ, Bender J, Mount DW, Jorgensen RA (2002) Analysis of histone acetyltransferase and histone deacetylase families of Arabidopsis thaliana suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Research **30:** 5036-5055

Pelaz S, Tapia-Lopez R, Alvarez-Buylla ER, Yanofsky MF (2001) Conversion of leaves into petals in Arabidopsis. Current Biology **11:** 182-184

Perani M, Ingram CJE, Cooper CS, Garrett MD, Goodwin GH (2003) Conserved SNH domain of the proto-oncoprotein SYT interacts with components of the human chromatin remodelling complexes, while the QPGY repeat domain forms homo-oligomers. Oncogene **22:** 8156-8167

Perry RP (2007) Balanced production of ribosomal proteins. Gene **401:** 1-3

Picard D, Salser SJ, Yamamoto KR (1988) A movable and regulable inactivation function within the steroid binding domain of the glucocorticoid receptor. Cell **54:** 1073-1080

Piques M, Schulze WX, Hohne M, Usadel B, Gibon Y, Rohwer J, Stitt M (2009) Ribosome and transcript copy numbers, polysome occupancy and enzyme dynamics in Arabidopsis. Molecular Systems Biology **5**

Proost S, Van Bel M, Sterck L, Billiau K, Van Parys T, Van de Peer Y, Vandepoele K (2009) PLAZA: a comparative genomics resource to study gene and genome evolution in plants. Plant Cell **21:** 3718-3731

Putterill J, Robson F, Lee K, Simon R, Coupland G (1995) The Constans Gene of Arabidopsis Promotes Flowering and Encodes a Protein Showing Similarities to Zinc-Finger Transcription Factors. Cell **80:** 847-857

Rashotte AM, Goertzen LR (2010) The CRF domain defines Cytokinin Response Factor proteins in plants. Bmc Plant Biology **10**

Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2006) A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proceedings of the National Academy of Sciences of the United States of America **103:** 11081-11085

Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396. Development **137:** 103-112

Salome PA, To JPC, Kieber JJ, McClung CR (2006) Arabidopsis response regulators ARR3 and ARR4 play cytokinin-independent roles in the control of circadian period. Plant Cell **18:** 55-69

Sarnowski TJ, Rios G, Jasik J, Swiezewski S, Kaczanowski S, Li Y, Kwiatkowska A, Pawlikowska K, Kozbial M, Kozbial P, Koncz C, Jerzmanowski A (2005) SWI3 subunits of putative SWI/SNF chromatin-remodeling complexes play distinct roles during Arabidopsis development. Plant Cell **17:** 2454-2472

Sarnowski TJ, Swiezewski S, Pawlikowska K, Kaczanowski S, Jerzmanowski A (2002) AtSWI3B, an Arabidopsis homolog of SWI3, a core subunit of yeast Swi/Snf chromatin remodeling complex, interacts with FCA, a regulator of flowering time. Nucleic Acids Research **30:** 3412-3421

Schmitz-Linneweber C, Small I (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends in Plant Science **13:** 663-670

Schruff MC, Spielman M, Tiwari S, Adams S, Fenby N, Scott RJ (2006) The *AUXIN RESPONSE FACTOR 2* gene of *Arabidopsis* links auxin signalling, cell division, and the size of seeds and other organs. Development **133:** 251-261

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Research **13:** 2498-2504

Sidaway-Lee K, Josse EM, Brown A, Gan YB, Halliday KJ, Graham IA, Penfield S (2010) SPATULA Links Daytime Temperature and Plant Growth Rate. Current Biology **20:** 1493-1497

Skirycz A, De Bodt S, Obata T, De Clercq I, Claeys H, De Rycke R, Andriankaja M, Van Aken O, Van Breusegem F, Fernie AR, Inzé D (2010) Developmental stage specificity and the role of mitochondrial metabolism in the response of Arabidopsis leaves to prolonged mild osmotic stress. Plant Physiology **152:** 226-244

Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Statistical Applications in Genetics and Molecular Biology **3:** Article 3

Srikanth A, Schmid M (2011) Regulation of flowering time: all roads lead to Rome. Cellular and Molecular Life Sciences **68:** 2013-2037

Su Y, Kwon CS, Bezhani S, Huvermann B, Chen C, Peragine A, Kennedy JF, Wagner D (2006) The N-terminal ATPase AT-hook-containing region of the Arabidopsis chromatin-remodeling protein SPLAYED is sufficient for biological activity. Plant J **46:** 685-699

Sweere U, Eichenberg K, Lohrmann J, Mira-Rodado V, Baurle I, Kudla J, Nagy F, Schafer E, Harter K (2001) Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. Science **294:** 1108-1111

Szerlong H, Hinata K, Viswanathan R, Erdjument-Bromage H, Tempst P, Cairns BR (2008) The HSA domain binds nuclear actin-related proteins to regulate chromatin-remodeling ATPases. Nature Structural & Molecular Biology **15:** 469-476

Tan QKG, Irish VF (2006) The Arabidopsis zinc finger-homeodomain genes encode proteins with unique biochemical properties that are coordinately expressed during floral development. Plant Physiol **140:** 1095-1108

Tang XR, Hou AF, Babu M, Nguyen V, Hurtado L, Lu Q, Reyes JC, Wang AM, Keller WA, Harada JJ, Tsang EWT, Cui YH (2008) The Arabidopsis BRAHMA chromatin-remodeling ATPase is involved in repression of seed maturation genes in leaves. Plant Physiol **147:** 1143-1157

Thaete C, Brett D, Monaghan P, Whitehouse S, Rennie G, Rayner E, Cooper CS, Goodwin G (1999) Functional domains of the SYT and SYT-SSX synovial sarcoma translocation proteins and co-localization with the SNF protein BRM in the nucleus. Human Molecular Genetics **8:** 585-591

To JPC, Haberer G, Ferreira FJ, Deruere J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2004) Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. Plant Cell **16:** 658-671

Ueno Y, Ishikawa T, Watanabe K, Terakura S, Iwakawa H, Okada K, Machida C, Machida Y (2007) Histone deacetylases and ASYMMETRIC LEAVES2 are involved in the establishment of polarity in leaves of Arabidopsis. Plant Cell **19:** 445-457

Usadel B, Nagel A, Steinhauser D, Gibon Y, Blasing OE, Redestig H, Sreenivasulu N, Krall L, Hannah MA, Poree F, Fernie AR, Stitt M (2006) PageMan: an interactive ontology tool to generate, display, and annotate overview graphs for profiling experiments. BMC Bioinformatics **7:** 535

van der Knaap E, Kim JH, Kende H (2000) A novel gibberellin-induced gene from rice and its potential regulatory role in stem growth. Plant Physiol **122:** 695-704

Van Leene J, Eeckhout D, Persiau G, Van De Slijke E, Geerinck J, Van Isterdael G, Witters E, De Jaeger G (2011) Isolation of transcription factor complexes from Arabidopsis cell suspension cultures by tandem affinity purification. Methods Mol Biol **754:** 195-218

Van Leene J, Stals H, Eeckhout D, Persiau G, Van De Slijke E, Van Isterdael G, De Clercq A, Bonnet E, Laukens K, Remmerie N, Henderickx K, De Vijlder T, Abdelkrim A, Pharazyn A, Van Onckelen H, Inzé D, Witters E, De Jaeger G (2007) A tandem affinity purification-based technology platform to study the cell cycle interactome in *Arabidopsis thaliana*. Molecular & Cellular Proteomics **6:** 1226-1238

Van Leene J, Witters E, Inzé D, De Jaeger G (2008) Boosting tandem affinity purification of plant protein complexes. Trends in Plant Science **13:** 517-520

Vert G, Walcher CL, Chory J, Nemhauser JL (2008) Integration of auxin and brassinosteroid pathways by Auxin Response Factor 2. Proceedings of the National Academy of Sciences of the United States of America **105:** 9829-9834

Wagner D, Meyerowitz EM (2002) SPLAYED, a novel SWI/SNF ATPase homolog, controls reproductive development in Arabidopsis. Current Biology **12:** 85-94

Wang L, Gu X, Xu D, Wang W, Wang H, Zeng M, Chang Z, Huang H, Cui X (2011a) miR396-targeted AtGRF transcription factors are required for coordination of cell division and differentiation during leaf development in Arabidopsis. J Exp Bot **62:** 761-773

Wang L, Hua D, He J, Duan Y, Chen Z, Hong X, Gong Z (2011b) *Auxin Response Factor2* (*ARF2*) and its regulated homeodomain gene *HB33* mediate abscisic acid response in *Arabidopsis*. PLoS Genetics **7:** e1002172

Wang WD, Cote J, Xue Y, Zhou S, Khavari PA, Biggar SR, Muchardt C, Kalpana GV, Goff SP, Yaniv M, Workman JL, Crabtree GR (1996a) Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. Embo Journal **15:** 5370-5382

Wang WD, Xue YT, Zhou S, Kuo A, Cairns BR, Crabtree GR (1996b) Diversity and specialization of mammalian SWI/SNF complexes. Genes & Development **10:** 2117-2130

Wang ZJ, Yuan TT, Yuan C, Niu YQ, Sun DY, Cui SJ (2009) LFR, which encodes a novel nuclear-localized Armadillo-repeat protein, affects multiple developmental processes in the aerial organs in Arabidopsis. Plant Mol Biol **69:** 121-131

Warner JR (1999) The economics of ribosome biosynthesis in yeast. Trends in Biochemical Sciences **24:** 437-440

Werner T, Schmülling T (2009) Cytokinin action in plant development. Current Opinion in Plant Biology **12:** 527-538

Wilson BG, Roberts CWM (2011) SWI/SNF nucleosome remodellers and cancer. Nature Reviews Cancer **11:** 481-492

Winter CM, Austin RS, Blanvillain-Baufume S, Reback MA, Monniaux M, Wu MF, Sang Y, Yamaguchi A, Yamaguchi N, Parker JE, Parcy F, Jensen ST, Li HZ, Wagner D (2011)

LEAFY Target Genes Reveal Floral Regulatory Logic, cis Motifs, and a Link to Biotic Stimulus Response. Developmental Cell **20:** 430-443

Wu JI, Lessard J, Olave IA, Qiu Z, Ghosh A, Graef IA, Crabtree GR (2007) Regulation of dendritic development by NeuronSpecific chromatin remodeling complexes. Neuron **56:** 94-108

Wu MF, Sang Y, Bezhani S, Yamaguchi N, Han SK, Li ZT, Su YH, Slewinski TL, Wagner D (2012) SWI2/SNF2 chromatin remodeling ATPases overcome polycomb repression and control floral organ identity with the LEAFY and SEPALLATA3 transcription factors. Proceedings of the National Academy of Sciences of the United States of America **109:** 3576-3581

Zemp I, Kutay U (2007) Nuclear export and cytoplasmic maturation of ribosomal subunits. Febs Letters **581:** 2783-2793

Zeng L, Zhou MM (2002) Bromodomain: an acetyl-lysine binding domain. Febs Letters **513:** 124-128

Zhao KJ, Wang WD, Rando OJ, Xue YT, Swiderek K, Kuo A, Crabtree GR (1998) Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. Cell **95:** 625-636

Zhou CH, Miki B, Wu KQ (2003) CHB2, a member of the SWI3 gene family, is a global regulator in Arabidopsis. Plant Mol Biol **52:** 1125-1134

5

Image: Ten-day-old Arabidopsis Col-0 seedling expressing *ARR4:GUS* after overnight staining.

Chapter 5: CYTOKININ RESPONSE FACTOR 3 promotes cell proliferation during Arabidopsis leaf growth

Liesbeth Vercruyssen[1,2], Giel Van Noorden[1,2,3], Nathalie Gonzalez[1,2], Boris Parizot[1,2], Megan Andriankaja[1,2], Liesbeth De Milde[1,2], Twiggy Van Daele[1,2], Tom Beeckman[1,2], and Dirk Inzé[1,2]

[1]Department of Plant Systems Biology, VIB, 9052, Ghent, Belgium

[2]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052, Ghent, Belgium

[3]Current Address: Research School of Biology, College of Medicine, Biology and Environment, The Australian National University, Canberra ACT 0200, Australia

L.V. conducted all experiments shown in the figures, i.e. leaf growth analyses, GUS staining and crosses. L.D.M. helped during the GUS stainings and T.V.D. in making the cell drawings. G.V.N. and B.P. provided all transgenic lines and constructs. M.A. shared AGRONOMICS1 tiling array expression data. N.G., T.B. and D.I. contributed useful comments and corrections.

ABSTRACT

Arabidopsis leaf development is characterized at the cellular level by the succession of five different but partially overlapping processes: primordium initiation, cell division, transition, cell expansion and meristemoid division. Spatial and temporal control is accomplished by a complex regulatory network of genes that when overexpressed or mutated affect one or more processes often resulting in changes in final leaf size. Here, overexpression of the transcription factor *CYTOKININ RESPONSE FACTOR 3* (*CRF3*) is reported to increase leaf growth by stimulating cell proliferation. Detailed leaf growth and cellular analysis suggest that CRF3 specifically acts to promote the rate of cell division during leaf development. In agreement, expression of *CRF3* is strongest when the leaves are fully proliferating. In addition, *CRF3* expression could be associated with the cell cycle arrest front and the leaves showed signs of enhanced developmental timing, suggesting a further role for CRF3 during the transition from cell proliferation to cell expansion. CRF3 function does not appear to be interconnected with that of the transcription factor GROWTH REGULATING FACTOR 5 (GRF5), although GRF5 was

previously shown to stimulate cell division in concert with cytokinins. Analysis of CRF3 function contributes to elucidating the complex growth regulatory network that governs leaf development in response to hormones.

INTRODUCTION

Arabidopsis (*Arabidopsis thaliana*) leaf primordia protrude from the side of the shoot apical meristem (SAM), with leaves 1&2 forming a decussate pattern together with the cotyledons followed by a spiral emergence of the younger leaves. The rod-shaped primordia subsequently establish polarity along the proximo-distal, medio-lateral, and dorso-ventral axis to grow out to mature leaves with flat lamina. Two processes that contribute to the determination of final leaf size are cell proliferation and cell expansion (Donnelly et al., 1999; Beemster et al., 2005; Gonzalez et al., 2012). The first days after emergence, cells of the leaf primordia are only dividing. Next, cell expansion starts from the tip of the leaf thereby quickly establishing the cell cycle arrest front, which remains at a relative constant position for a few days, and then progresses rapidly towards the base of the leaf to disappear abruptly (Kazama et al., 2010; Andriankaja et al., 2012). From then on, leaf growth is mainly driven by cell expansion due to turgor and cell wall loosening (Cosgrove, 2005).

Cytokinins are implicated in numerous processes during plant development, including shoot apical meristem establishment, chloroplast development and leaf growth (Mok, 1994; Hwang et al., 2012). Judging from the strong reduction in leaf size and cell number in several cytokinin signaling or metabolism mutants, cytokinins are important for leaf cell proliferation (Werner et al., 2003; Nishimura et al., 2004; Riefler et al., 2006; Argyros et al., 2008). Consistently, localized degradation of cytokinins by the expression of *CYTOKININ OXIDASES/DEHYDROGENASE 3* (*CKX3*) under control of the *AINTEGUMENTA (ANT)* promoter, revealed that cytokinins specifically stimulate the duration of the cell proliferation phase (Holst et al., 2011). Furthermore, expression of *CYCB1;1* and *CYCD3* cell cycle genes was shown to be enhanced by cytokinin treatment (Riou-Khamlichi et al., 1999; Dewitte et al., 2007; Vercruyssen et al., chapter 3).

However, additional molecular information on how cytokinins affect cell division during leaf development is missing.

Cytokinin signaling is mediated by a two-component histidine-to-aspartate (His-to-Asp) phosphorelay that initiates with the autophosphorylation of the receptor ARABIDOPSIS HISTIDINE KINASES, AHK2, AHK3 and AHK4/CRE1/WOL (Mahonen et al., 2000; Inoue et al., 2001; Suzuki et al., 2001a; Ueguchi et al., 2001; Yamada et al., 2001), followed by phosphotransfer to ARABIDOPSIS HIS PHOSPHOTRANSFER proteins (AHP1-5) (Miyata et al., 1998; Suzuki et al., 1998; Suzuki et al., 2000), and ending with the phosphorylation of two types of ARABIDOPSIS RESPONSE REGULATORS (A-type and B-type ARRs) (Brandstatter and Kieber, 1998; Sakai et al., 1998; Imamura et al., 1999; Lohrmann et al., 1999; D'Agostino et al., 2000; Hwang and Sheen, 2001; Suzuki et al., 2001b; Hutchison et al., 2006; Dortay et al., 2008; Hwang et al., 2012). The 11 B-type ARRs (ARR1, 2, 10-14, 18-21) function as transcription factors to induce the expression of the primary cytokinin response genes, including the ten A-type *ARRs* (*ARR3-9, 15-17*) (Mason et al., 2005; Kim et al., 2006; Taniguchi et al., 2007; Argyros et al., 2008; Brenner et al., 2012). The latter negatively feed-back on cytokinin signaling, most likely at the level of the AHPs (Kiba et al., 2003; To et al., 2004; Dortay et al., 2006; Lee et al., 2008).

The CYTOKININ RESPONSE FACTOR (CRF) family was initially described as a small subgroup within the ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (ERF) family of APETALA 2/ERF (AP2/ERF) transcription factors comprising six members (CRF1-CRF6) (Rashotte et al., 2006). Extensive phylogenetic analysis revealed the presence of a novel sequence termed the CRF domain, in 6 additional Arabidopsis proteins (CRF7, CRF8 and B1-B4), as well as in proteins of rice, poplar and tomato (Rashotte and Goertzen, 2010). The amino-terminal CRF domain is inextricably associated with a distinctive AP2/ERF DNA binding domain, constituting the central part of the protein, whereas the presence of a more widely distributed putative MAP kinase phosporylation site in the carboxy-terminal part is restricted to a subset of CRFs (CRF1-CRF6). In addition, CRF1 to CRF8 contain a so-called TEH domain in front of the CRF domain, that distinguishes clade A from clade B CRF proteins (Rashotte and Goertzen, 2010).

CRF2 and *CRF5* were recently appointed by meta analysis of eight microarrays to belong to the top 20 most robust genes regulated by cytokinin, and also *CRF6* transcription was shown to be induced upon cytokinin treatment (Rashotte et al., 2006; Brenner et al., 2012). However, *CRF1*, *CRF3* and *CRF4* were not transcriptionally responsive to cytokinin, but all six CRF proteins relocalize to the nucleus upon cytokinin application in protoplasts (Rashotte et al., 2006). This movement was demonstrated for CRF2 to be dependent on phosphorylation and on the presence of the AHKs and AHPs, but not the ARRs (Rashotte et al., 2006). In addition, CRF1-8 were shown to homo- and heterodimerize and interact with the AHPs through the CRF domain (Cutcliffe et al., 2011). Single and double mutations of *crf1* to *crf6* resulted in aberrant cotyledon development characterized by deformed edges and reduced greening with a penetrance lower than 10%. This phenotype was more pronounced in triple *crf1/2/5* and *crf2/3/6* mutants and shown to be due to a predominant reduction in cell size, indicating that CRFs function redundantly in cotyledon expansion. Moreover, transcript profiling of these triple mutants revealed that CRFs regulate transcription of a large portion of cytokinin-response genes, many of them which are also differentially regulated by B-type ARRs. Taken together, this places the CRF signaling module as a parallel pathway branching from the AHPs, that regulates a significant part of cytokinin responsive gene expression during seedling development (Rashotte et al., 2006).

Besides a functionally redundant role during cotyledon development for CRF1, CRF2, CRF3, CRF5 and CRF6, and during embryo development for CRF5 and CRF6, no specific functions for the CRFs could be inferred from the different mutants (Rashotte et al., 2006). However, overexpression of *CRF2* enhanced chloroplast division in mesophyll cells, similarly to cytokinin treatment (Okazaki et al., 2009). This is in agreement with the observed white spots in cotyledons of *crf1/2/5* mutants, suggesting that the positive role of cytokinins in chloroplast development could be, at least in part, mediated through *CRF2*. Furthermore, *CRF2* is under direct transcriptional control of MONOPTEROS (MP), an auxin-responsive transcription factor important for embryonic root meristem initiation, linking cytokinin and auxin signaling pathways during embryogenesis (Schlereth et al., 2010). Roots overexpressing *CRF3* sporadically developed callus-like tissue in the absence of exogenous hormones and *CRF3* expression was shown to be

strongly induced in aerial and root explants during callus initiation, a process thought to originate in pericycle cells (Atta et al., 2009; Sugimoto et al., 2010; Xu et al., 2012). Furthermore, *CRF3* was identified as an auxin induced gene during lateral root initiation, which starts with asymmetric pericycle cell divisions (Vanneste et al., 2005). In accordance with a positive role in lateral root formation, *crf3* loss of function and *CRF3* overexpressing plants have a decreased and increased number of lateral root primordia, respectively (Van Noorden et al., unpublished). In addition, *CRF1*, *CRF2* and *CRF3* were found to be directly repressed by PHYTOCHROME INTERACTING FACTOR 3-LIKE 5 (PIL5/PIF1), that inhibits seed germination in the dark by simultaneously decreasing abscisic acid levels and increasing gibberellin responsiveness and levels (Oh et al., 2004; Oh et al., 2006; Oh et al., 2007; Oh et al., 2009). Also tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tobacum*) *CRFs* were demonstrated to be cytokinin responsive and/or regulated by other hormones, biotic or abiotic factors (Zhou et al., 1997; Park et al., 2001; Gu et al., 2002; Ham et al., 2006; Shi et al., 2012).

It becomes clear that the CRFs are important players in embryo, root and shoot developmental processes, as integrators of hormonal responses and external stimuli. To gain further insight in the role of CRF3 during vegetative shoot development, leaf growth of *crf3* loss of function mutants and plants overexpressing *CRF3* was extensively analyzed at the phenotypic level. In addition, the expression pattern of CRF3 during leaf development was examined, as well as the relationship with the cell division-promoting transcription factor GROWTH REGULATING FACTOR 5 (GRF5).

RESULTS

CRF3 overexpression enhances shoot growth

To investigate if CRF3 acts beyond the root system, rosette growth was analyzed of loss and gain of function lines described by Van Noorden et al. (unpublished). Wild-type Col-0, *crf3* and *35S:CRF3* plants were grown *in vitro* in both long day conditions (16h light/8h dark) and under continuous light. Rosettes were harvested 21 days after stratification (DAS), and total leaf area was determined.

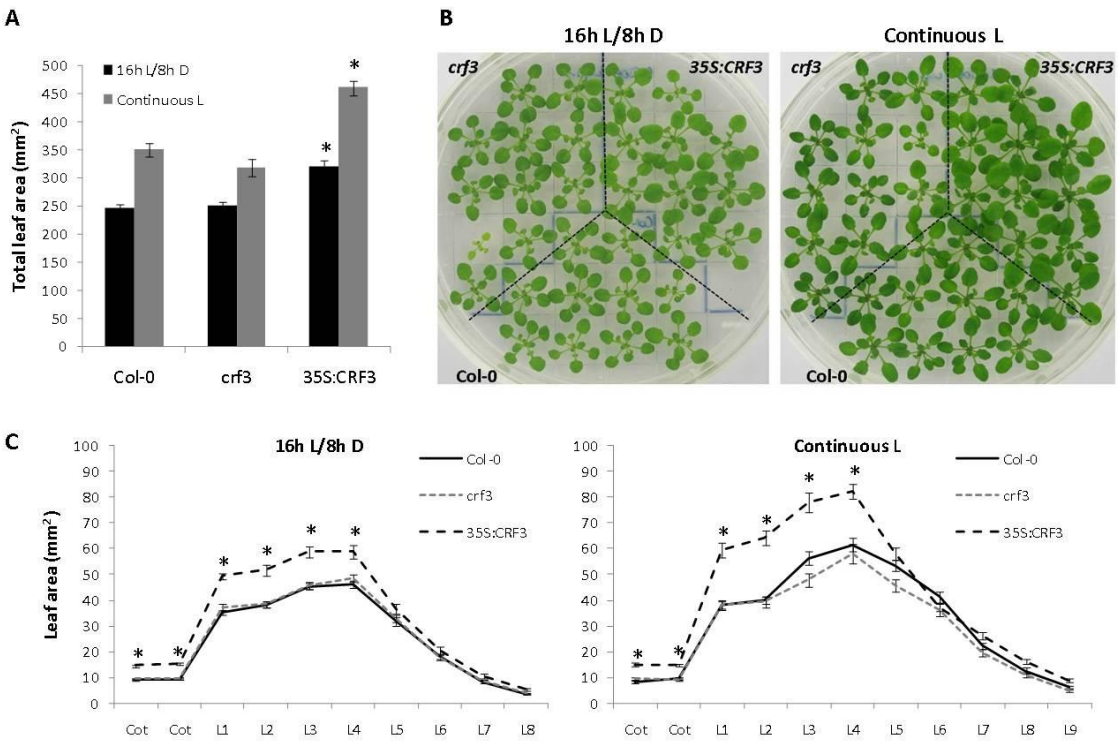


Figure 1. Overexpression of *CRF3* enhances shoot growth in long day and continuous light conditions.

A, Total rosette area, calculated from leaf series made at 21 days after stratification (DAS) from wild-type (Col-0), *crf3* and *35S:CRF3* plants, grown in long day (16h light/8h dark) or continuous light conditions. B, Rosettes of 21-day-old wild-type, *crf3* and *35S:CRF3* plants, grown in long day or continuous light conditions. C, Individual area of cotyledons (Cot) and leaves 1 to 9 (L1-L9), measured from leaf series of plants grown as described in A. Error bars indicate SE (n = 16). * Significantly different from the wild type ($P < 0.01$, Student's *t* test).

Whereas *crf3* rosette growth was indistinguishable from Col-0, *35S:CRF3* total rosette area was significantly increased compared to Col-0 in both light conditions (Fig. 1, A and B). Leaf series were made from 21-day-old rosettes to allow for a more detailed analysis of individual leaf sizes. In day/night as well as continuous light conditions, the size of *35S:CRF3* cotyledons and leaves 1, 2, 3 and 4 was increased, while the younger leaves were equal in size compared to wild-type leaves (Fig. 1, C). Comparison of the relative increases in total rosette size and individual leaf size in continuous versus long day conditions, showed the lack of significant differences between *35S:CRF3* and Col-0 (Supplemental Fig. 1, A and B), indicating that the prolonged light period enhances leaf

growth to the same extent in both lines. Furthermore, both leaf width and leaf length were increased due to *CRF3* overexpression (Supplemental Fig. 1, C and D). The leaves of *crf3* plants were not significantly different from Col-0, in agreement with the fresh weight measurements (Fig. 1, C) and previous observations (Rashotte et al., 2006), indicating that CRF3 most likely functions redundantly with other CRFs during leaf growth. However, overexpression of *CRF3* enhances leaf growth in a way that is polarity independent and not sensitized by continuous light conditions.

35S:CRF3 affects early leaf growth

Recently, at least five different cellular processes have been described that are supported by experimental data to contribute to the formation of larger leaves: (I) the presence of more cells in the leaf primordium; (II) an increase in cell division rate; (III) an increase in the duration of cell division; (IV) an increase in cell expansion and (V) an enhanced meristemoid division (Gonzalez et al., 2012). To determine which process causes the increase in *35S:CRF3* leaf size, the blade area of leaves 1&2 was measured daily, starting from 4 DAS when the leaf pirmordia can first be distinguished with use of the stereomicroscope, until 21 DAS. No significant difference between wild-type and *35S:CRF3* leaf size could be observed at 4 DAS, with an average primordium size smaller than 0,02 mm^2 (Fig. 2, A). This makes suspect that an equal amount of cells is recruited from the SAM to initiate the primordium. However, starting from 5 DAS, *35S:CRF3* leaves 1&2 became larger compared to wild-type leaves (Fig. 2, A). The enhanced growth was significant for most of the early time points, for which blade expansion of leaves 1&2 was shown to be proportional to the increase in cell number (Beemster et al., 2005). This suggests that CRF3 acts during the proliferation phase of leaf development.

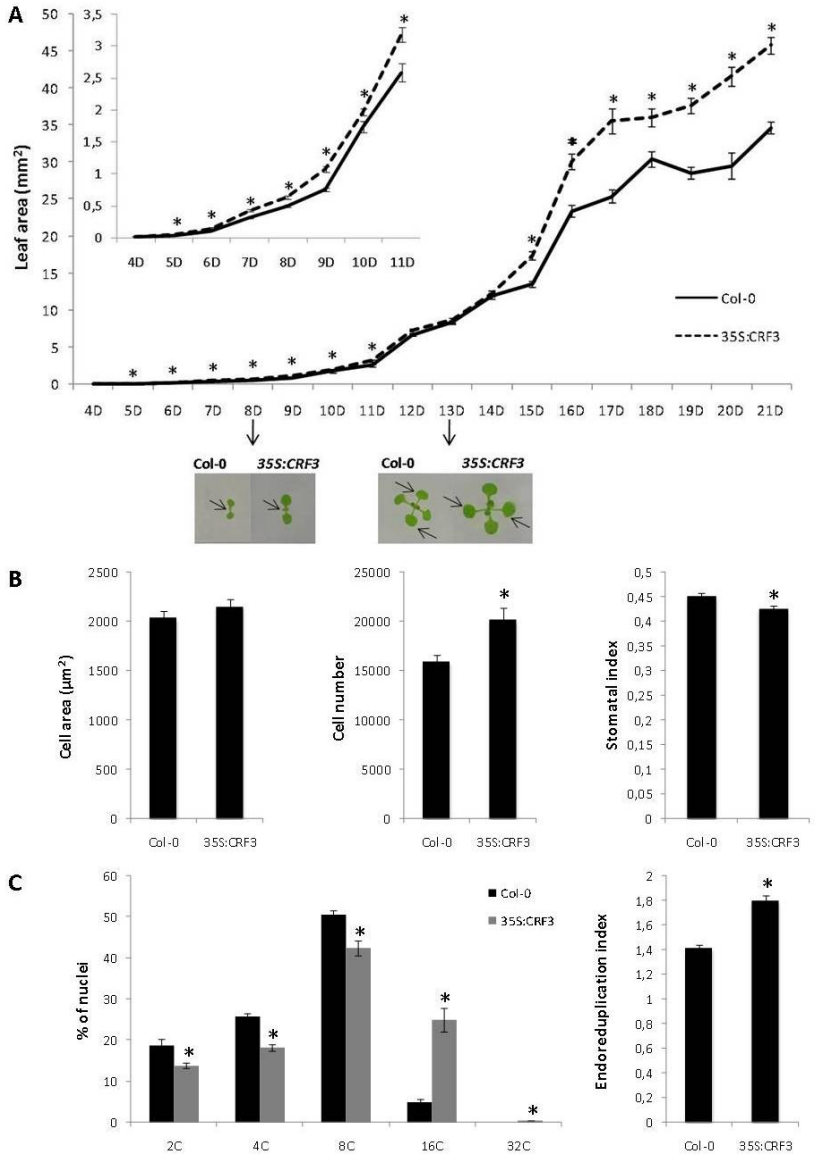


Figure 2. Overexpression of *CRF3* stimulates cell proliferation.

A, Leaf blade area of leaves 1&2 of wild-type (Col-0) and *35S:CRF3* plants measured daily from 4 to 21 days after stratification (D). The inset shows an enlargement of the graph from 4 to 11 days. Error bars are SE ($n \geq 19$). * Significantly different from the wild type ($P < 0.05$, Student's *t* test). Pictures of wild-type and *35S:CRF3* seedlings were taken at 8 and 13 days. Arrows indicate leaves 1&2. B, Average pavement cell area, cell number and stomatal index of wild-type and *35S:CRF3* leaves 1&2 at 21 days, calculated from cell drawings as described in material and methods. Stomatal index: number of guard cells relative to total cell number. Error bars indicate SE ($n = 10$). * Significantly different from the wild type ($P < 0.05$, Student's *t* test). C, Ploidy analysis of wild-type and *35S:CRF3* leaves 1&2 at 21 days. The percentages of 2C, 4C, 8C, 16C and 32C nuclei are presented. Endoreduplication index: average number of endocycles undergone by a typical nucleus, calculated as 1*4C+2*8C+3*16C+4*32C. Error bars are SE ($n = 3$). * Significantly different from the wild type ($P < 0.05$, Student's *t* test).

CRF3 promotes leaf cell division and development

To confirm the mechanism driving *35S:CRF3* enhanced leaf growth, cellular parameters were determined from 21-day-old leaves 1&2. Whereas average epidermal cell area of *35S:CRF3* leaves was similar to that of wild-type cells, average cell number was significantly increased by 27% (Fig. 2, B). In addition, the fraction of guard cells relative to the total cell number, defined by the stomatal index, was decreased in *35S:CRF3* leaves (Fig. 2, B), most likely as a consequence of the increased amount of pavement cells. Taken together, overexpression of *CRF3* stimulates cell division, but does not seem to interfere with cell expansion or meristemoid division.

Next, ploidy levels of leaves 1&2 at 21 DAS were analyzed, by measurement of the nuclear DNA content with flow cytometry. The majority of the cells had a 8C content both in wild-type and *35S:CRF3* leaves (Fig. 2, C), an indication of ongoing endoreduplication. Only a small fraction of 16C nuclei were present in wild-type leaves, while about one fourth of the cells had 16C nuclei and some cells up to 32C nuclei in *35S:CRF3* leaves (Fig. 2, C). This led to a concomitant reduction in 2C, 4C and 8C content compared to wild-type leaves. As a result, a significant increase could be observed in the endoreduplication index of *35S:CRF3* leaves 1&2, that represents the average number of endocycles undergone by a typical nucleus (Fig. 2, C). Although postulated and often described to be tightly linked, the increase in endoreduplication does not seem to correlate with an increase in average cell size for *35S:CRF3* leaves, at least not at 21 DAS. An alternative explanation could be provided by the possibility that *35S:CRF3* leaves are at an enhanced developmental stage at 21DAS. This could be ascribed to an enhanced rate of cell division that results in an earlier exit from the proliferation phase and a simultaneous earlier onset of endoreduplication. Cell division ceased in wild type around 12 DAS and cell expansion mainly accounted for the growth of the leaf blade between days 12 and 19 (Beemster et al., 2005). The strong increase in leaf blade size between 15 and 16 DAS could actually be observed already one day earlier in *35S:CRF3* leaves 1&2 (Fig. 2, A), arguing in favor of a faster development of *35S:CRF3* compared to Col-0 leaves.

CRF3 expression precedes and coincides with the mitotic arrest front

The detailed analysis of *35S:CRF3* leaves suggests that CRF3 functions to stimulate cell proliferation in a developing leaf. Therefore, the expression pattern of *CRF3* is likely associated with the zone of cell proliferation in the leaf. To investigate this, the *CRF3* promoter was used to drive *GUS* expression and two independent transformed lines, *CRF3:GUS-1* and *CRF3:GUS-2*, were analyzed for GUS staining (Van Noorden et al., unpublished). In parallel, plants expressing *CYCB1;1:D-Box-GUS-GFP* (*CYCB1;1:DB-GUS*) were examined (Eloy et al., 2011). *CYCB1;1:DB-GUS* is a marker for actively dividing cells, which enables to delineate the proliferation zone during leaf development (Colón-Carmona et al., 1999).

The plants were harvested from 6 to 13 DAS to analyze GUS staining in leaves 1&2 during the subsequent phases of cell proliferation, transition and cell expansion. At days 6 and 7, when the majority of the cells are proliferating as indicated by *CYCB1;1:DB-GUS* staining, *CRF3:GUS* could be observed in the distal part of the leaves (Fig. 3, A-B and E-F). This indicates that *CRF3* is expressed in dividing cells, but not in the dividing cells at the leaf base. Subsequently, at 8 and 9 DAS cell division ceases at the tip of the leaf where cell expansion starts, while *CRF3:GUS-1* was still observed in the distal leaf part and extended further down the middle of the leaf. The proximal leaf cells were still devoid of *CRF3:GUS-1* expression (Fig. 3, C-D and G-H). Thus, *CRF3* is expressed in the dividing cells located at the distal end of the division zone and in cells that most likely initiated expansion. Between days 9 and 10 the cell cycle arrest front rapidly moves down the leaf (Andriankaja et al., 2012), resulting in *CYCB1;1:DB-GUS* staining predominantly in the basal cells of the leaves during days 10 and 11 (Fig. 3, K and L). At 10 DAS a *CRF3:GUS-1* pattern similar to that of 9 DAS was detected, with GUS staining in the most distal dividing cells of the division zone and in the expanding cells. However, expression started to disappear near the tip of the leaf (Fig. 3, I). *CRF3:GUS-1* staining became more and more restricted to the leaf halves excluding the longitudinal axis at 11 DAS (Fig. 3, J). From days 11 to 12 virtually all *CYCB1;1:DB-GUS* staining had disappeared, concomitant with the reported abrupt abolishment of the mitotic arrest front (Andriankaja et al., 2012), whereas faint and dispersed *CRF3:GUS-1* staining was present

near the side of the leaves that had disappeared almost completely at 13 DAS (Fig. 3, M-N and O-P).

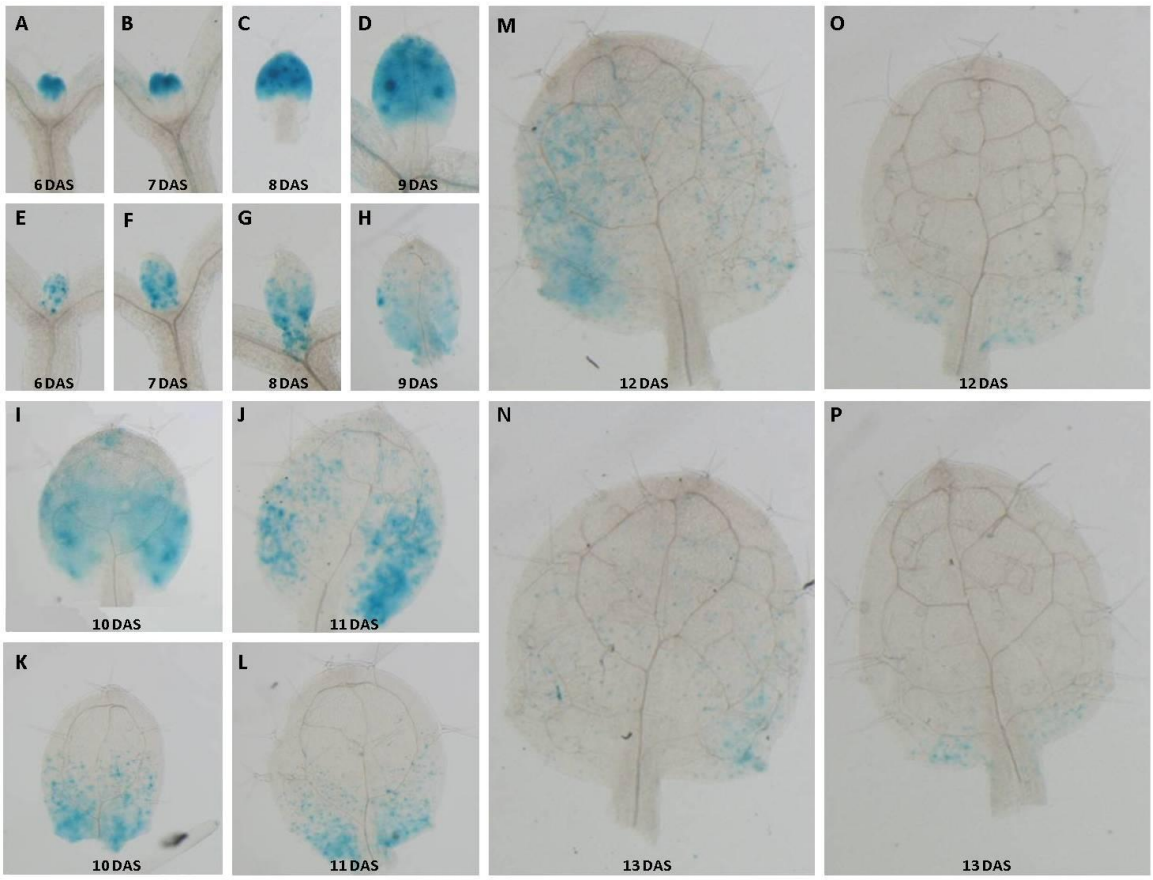


Figure 3. *CRF3* expression during development of leaves 1&2.

CRF3:GUS-1 and *CYCB1;1:DB-GUS* control plants were harvested from 6 to 13 DAS and GUS stained for 8h, after which leaves 1&2 were photographed. A-D, I-J, M and N: *CRF3:GUS*. E-H, K-L, O and P: *CYCB1;1:DB-GUS*.

A similar GUS staining pattern was observed in the *CRF3:GUS-2* leaves 1&2, with the strongest expression observed at days 6 and 7. The recently published transcriptome sets of leaf 3 from days 8 to 13, covering the subsequent phases from proliferation to expansion, were used to check transcript levels of *CRF3* (Andriankaja et al., 2012). In agreement with the GUS staining, the normalized expression of *CRF3* is highest when the leaf is fully proliferating, and gradually decreases during the transition until all cells

stopped dividing (Supplemental Fig. 2, A). In addition, a comparable pattern of normalized transcript data could be observed for *CRF1*, *2*, *4*, *5*, *10* and *11* (Supplemental Fig. 2, B). Furthermore, no *CRF3:GUS* was detected in the SAM, although the RAM showed intense staining (Supplemental Fig. 3).

Taken together, *CRF3* is expressed strongly in dividing cells during early leaf development in a region that seems to precede the position of the mitotic arrest front. In addition *CRF3* is expressed in the cells that started the expansion process, but expression decreases rapidly to disappear when proliferation has ceased in the leaf.

CRF3 and GRF5 work independently to stimulate leaf growth

Overexpression of *CRF3* enhances cell proliferation and *CRF3* is expressed in dividing cells before the transition as well as in a broad region coinciding with the transition zone. Overexpression of *GRF5* also increases leaf size, by increasing both the rate and duration of cell proliferation during leaf development thereby delaying the transition to cell expansion (Horiguchi et al., 2005; Gonzalez et al., 2010; Vercruyssen et al., chapter 3). Furthermore, GRF5 and cytokinins were demonstrated to work together to promote leaf growth by synergistically increasing *CYCB1;1* expression in young leaves (Vercruyssen et al., 2011; Vercruyssen et al., chapter 3). Therefore, the possibility exists that CRF3 and GRF5 functions are interconnected during leaf development. To investigate this, the overexpression of both genes was combined to test if it leads to a further increase in leaf growth and fresh weight production.

Homozygous *35S:CRF3* and *35S:GRF5* plants were crossed and in addition, both lines were simultaneously crossed with wild-type Col-0 plants, resulting in heterozygous lines that were used as appropriate controls. At 21 DAS *35S:CRF3*, *Col-0 x 35S:CRF3* and *35S:GRF5* rosettes exhibited a similar increase in leaf fresh weight when compared to wild-type plants (Fig. 4, A). Fresh weight of *Col-0 x 35S:GRF5* plants was not different from wild-type fresh weight, however, detailed analysis of individual leaf areas revealed that the cotyledons and leaves 1&2 were increased in size, while the younger leaves were similar in size or smaller than wild-type leaves (Fig. 4, A-C). The gene dosage effect of

35S:GRF5 and leaf series profile are in agreement with previous observations (Gonzalez et al., 2010; Vercruyssen et al., 2011).

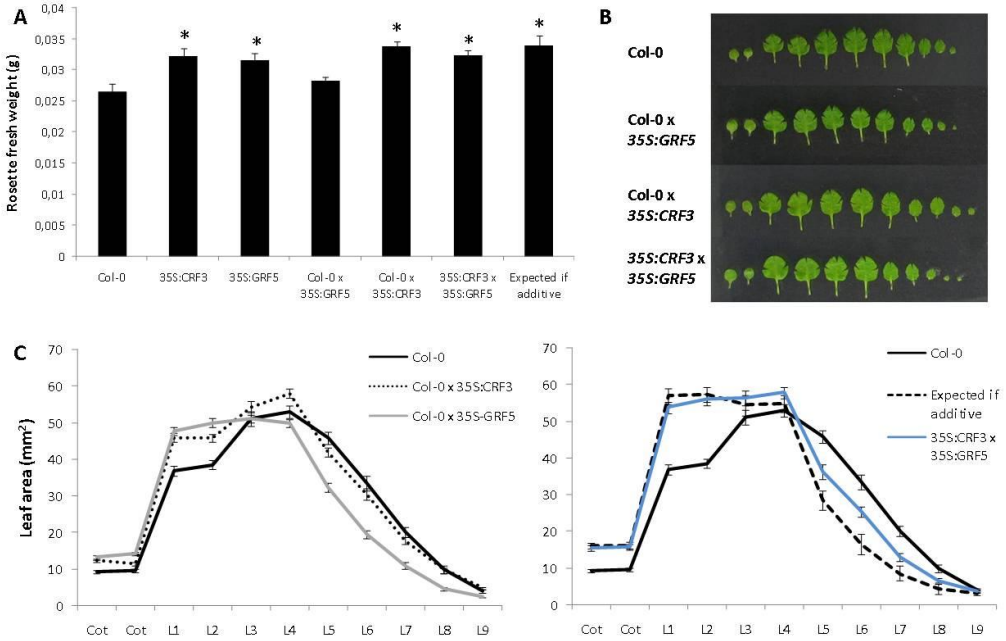


Figure 4. Combined overexpression of *CRF3* and *GRF5* additively enhances shoot growth.

A, Rosette fresh weight of wild-type (Col-0) plants, homogygous *35S:CRF3* and *35S:GRF*5 plants, heterozygous parents and crossed plants at 21 DAS. Error bars are SE ($n \geq 22$). * Significantly different from the wild type ($P < 0.01$, Student's *t* test). B, Leaf series of 21-day-old plants. C, Individual area of cotyledons (Cot) and leaves 1 to 9 (L1-L9), measured from leaf series of plants at 21 DAS. Error bars indicate SE ($n \geq 14$). Values expected if additive were calculated as the sum of the single heterozygous lines minus the wild type.

To determine the effect of combined *CRF3* and *GRF5* overexpression, expected values for additive effects on leaf fresh weight and leaf area were calculated. Additive phenotypes correspond to the sum of the individual phenotypes of the heterozygous parent plants and usually indicate that the genes function in independent pathways. As such, *35S:CRF3 x 35S:GRF5* plants had a fresh weight that did not significantly differ from the expected fresh weight for an additive effect (Fig. 4, A). In accordance, *35S:CRF3 x 35S:GRF5* individual leaf sizes equaled the expected values for additive effects in both leaf width and leaf length directions (Fig. 4, B and C; Supplemental Fig. 4,

A and B). As a result, a strong increase in size of the cotyledons and leaves 1&2 of approximately 65% and 45% compared to wild type, respectively, could be attained (Fig. 4, B and C). Relative transgene expression levels in *35S:CRF3 x 35S:GRF5* shoots were similar to those of the heterozygous parents, indicating the absence of mutually induced changes in expression levels (Supplemental Fig. 4, C). In conclusion, *CRF3* and *GRF5* most likely exhibit an independent mode of action through distinct pathways during leaf growth.

DISCUSSION

The CRFs were originally identified as primary cytokinin response genes, that in turn regulate transcription of a large part of the cytokinin response similar to B-type ARRs, most likely through interaction with the AHPs (Rashotte et al., 2006; Cutcliffe et al., 2011).

By mutant analysis a role for CRF3 during cotyledon and juvenile leaf growth was demonstrated previously and shown to be functionally redundant with CRF2 and CRF6 (Rashotte et al., 2006). In agreement, no leaf phenotype could be observed in *crf3* single mutant plants under our growth conditions. Overexpression of *CRF3* on the other hand increased the size of the cotyledons and leaves, which was shown for leaves 1&2 to be due to an increase in the number of cells, while cell size was not affected. This is somewhat contradictory with the observation in the *crf1/2/5* triple mutant. The decrease in cotyledon area of around 96% was ascribed predominantly to the decrease in cell size, however, cell numbers were also reduced by almost 30% (Rashotte et al., 2006). Moreover, the cells in cotyledons and true leaves can be differentially affected, because a similar reduction in cotyledon cell size was observed in the *ahk2/3/4* triple mutant, while mature leaves contained a reduction in cell numbers that was in some cases even compensated for by an increase in cell size (Nishimura et al., 2004; Rashotte et al., 2006; Riefler et al., 2006). Furthermore, a stimulating role for CRF3 during cell proliferation could also be inferred from the fact that *CRF3* expression is strongly associated with the initiation of callus tissue, a process that requires extensive cell division (Xu et al., 2012).

Several lines of evidence support the hypothesis that CRF3 specifically enhances the rate of cell division. First, *CRF3:GUS* expression was absent from the SAM and *35S:CRF3* leaf primordia were equal in size to those of wild type at the earliest time point measured, indicating that most likely not more cells are recruited from the SAM for primordium initiation. Second, *CRF3* expression is strongest during the proliferation phase of leaf development and *35S:CRF3* leaves 1&2 become larger already early after primordium establishment when leaf growth is driven solely by cell division. Third, the *35S:CRF3* endoreduplication index is higher compared to wild type at 21 DAS, arguing in favor of a faster leaf development. As a consequence, the increase in leaf size without enhanced cell expansion can only be accomplished by an increase in the rate of cell proliferation.

Interestingly, *CRF3:GUS* could also be detected in cells at the mitotic arrest front and in the zone above it containing cells that most likely have started expansion. When all cells ceased proliferating, *CRF3:GUS* staining had also disappeared. Therefore, *CRF3* expression seems to be associated with the mitotic arrest front, suggesting a function during the transition from cell proliferation to cell expansion. It was demonstrated recently that chloroplast development is important for the correct regulation of the onset of cell expansion (Andriankaja et al., 2012). CRF3 could function to stimulate chloroplast division, as was reported for CRF2, and thereby affect the timing of the transition (Okazaki et al., 2009). More detailed analysis of *CRF3:GUS* staining along the transverse leaf axis will reveal if *CRF3* expression is correlated with the presence of chloroplasts in the mesophyll. Alternatively, CRF3 could act around the transition zone to control the cell division rate in the more proximally located cells.

The question remains whether CRF3 is integral part of the cytokinin signaling pathway that stimulates leaf cell proliferation. *CRF3* was not transcriptionally regulated by cytokinins, but the protein translocates to the nucleus upon cytokinin treatment and interacts with four of the five AHPs (Rashotte et al., 2006; Cutcliffe et al., 2011). On the other hand, *CRF3* is transcriptionally induced by auxin, at least in the root, and also *CRF2* was shown to be a target of the auxin-response factor MP (Vanneste et al., 2005; Schlereth et al., 2010). Moreover, the induction of *CRF3* during callus initiation is accompanied by the upregulation of genes from the auxin signaling pathway, while virtually no positive regulators of cytokinin signaling were identified (Xu et al., 2012).

Cytokinin and auxin function antagonistically during many processes with high cytokinin/auxin concentration promoting proliferation and low cytokinin/auxin levels promoting differentiation, although they often can-not exert their effects without each other (Su et al., 2011). Thus high auxin concentrations promote primordium establishment rather than outgrowth, and cell expansion rather than proliferation during leaf development, although auxin is also needed for proper cell division. This is illustrated for example by a reduction in cell numbers, size and endoreduplication in leaves with reduced AUXIN BINDING PROTEIN 1 (ABP1) activity (Braun et al., 2008; Scarpella et al., 2010). Therefore, we cannot exclude that CRF3 function is directed by auxin besides cytokinin during leaf development, and it is possible that CRF3 functions to integrate the response to both hormones.

Numerous genes have been described that enhance leaf growth when overexpressed or mutated and *CRF3* can now be added to this list (Gonzalez et al., 2009; Krizek, 2009; Gonzalez et al., 2012). Overexpression of *GRF5* and its interacting partner *ANGUSTIFOLIA 3/GRF INTERACTING FACTOR 1* (*AN3/GIF1*) also results in an increase in leaf size due to an increase in both the duration and the rate of cell division (Horiguchi et al., 2005; Lee et al., 2009; Gonzalez et al., 2010; Horiguchi et al., 2011; Vercruyssen et al., chapters 3 and 4). AN3 is a transcriptional co-activator that regulates target gene transcription in complex with GRFs or other transcription factors by association with SWI/SNF chromatin remodeling complexes (Horiguchi et al., 2005; Vercruyssen et al., chapter 4). Although *CRF2* was shown to be a putative direct transcriptional target of AN3, not GRF5 but rather another transcription factor would work together with AN3 in promoting *CRF2* transcription (Vercruyssen et al., chapter 4). Likewise, we show that CRF3 and GRF5 probably do not function in the same pathway, since combined overexpression of *CRF3* with *GRF5* did not result in synergistic but in additive effects on leaf growth. Remarkably, during dark-repression of germination PIF1 was shown to directly repress *GRF5* and *CRF3*, arguing in favor for the stimulation of similar processes by GRF5 and CRF3 through distinct parallel pathways (Oh et al., 2009). Thus, although GRF5 was shown to act in concert with cytokinins, this is probably not accomplished through CRF3. Taken together, whereas GRF5 positively regulates the rate and duration of cell proliferation, CRF3 most likely stimulates the cell division rate,

thereby fastening development possibly resulting in a negative effect on the duration of cell division. These different mechanisms could be a reflection of their independent mode of action.

Further research is needed to shed light on the precise regulation of CRF3 and its downstream targets. This will potentially broaden our understanding of the interplay between auxin and cytokinin during leaf development, of which a lot remains to be uncovered. Furthermore, altered growth can be observed in both the root and the shoot when *CRF3* is overexpressed and recently the transcripts of *35S:CRF3* plants during lateral root initiation were profiled (Van Noorden et al., unpublished). Since optimal growth of a plant requires a proper shoot to root balance, it would be very interesting to compare differentially expressed genes between the root and the shoot, potentially allowing for the identification of molecular players involved in the tight coordination of root and shoot growth.

MATERIALS AND METHODS

Plant material and growth conditions

35S:CRF3, crf3, CRF3:GUS-1 and *CRF3:GUS-2* plants were kindly provided by Giel Van Noorden (Van Noorden et al., unpublished). *35S:GRF5* seeds were a kind gift of Prof. Dr. Hirokazu Tsukaya (Horiguchi et al., 2005). *CYCB1;1:DB-GUS* plants were kindly provided by Dr. Nubia Eloy (Eloy et al., 2011). All plants are in the *Arabidopsis thaliana* (L.) Heyhn. ecotype Columbia (Col-0) background.

For in vitro experiments, seeds were sown in sterile plates containing half-strength Murashige and Skoog (½MS) medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.8% agar. The plates were sealed and put in a tissue culture room at 21°C under a 16h day/8h night regime or under long day (16h day/8h night) conditions (50 μmol m^{-2} s^{-1}).

Growth measurements

To measure rosette leaf parameters, 21-day-old *in vitro* grown seedlings were harvested and individual leaves (cotyledons and rosette leaves) were dissected and photographed, after which leaf areas were measured with the ImageJ software (http://rsb.info.nih.gov/ij/). Rosette areas were calculated as the sum of the individual leaf areas.

For the time-course of leaf area analysis, leaves 1&2 were harvested daily from 4 to 21 DAS. The youngest leaves were cleared in 100% and 70% ethanol and mounted on microscopy slides in 100% lactic acid, while the older leaves were put on an agar plate. Next, the leaves were photographed and the area was determined with ImageJ.

Cellular parameters were determined for leaves 1&2 at 21 DAS after clearing in ethanol and mounting on microscopy slides in lactic acid. Drawings of the abaxial epidermis were made for five leaves with a microscope (Leica) fitted with a drawing tube and a differential interference contrast (DIC) objective. Average cell areas and corresponding leaf areas were measured with ImageJ (http://rsb.info.nih.gov/ij/), from which cell numbers were calculated. The stomatal index was defined as the percentage of stomata among all cells.

Ploidy analysis

For flow cytometry analysis, 21-day-old leaves 1&2 from plants grown *in vitro* were chopped with a razor blade in 200 µL of Cystain UV Precise P Nuclei Extraction buffer (Partec), followed by the addition of 800 µL of staining buffer and filtering through a 50-µm filter. Nuclei were analyzed with the Cyflow MB flow cytometer (Partec) and the corresponding FloMax software. The endoreduplication index was calculated as %4C + 2 × %8C + 3 × %16C.

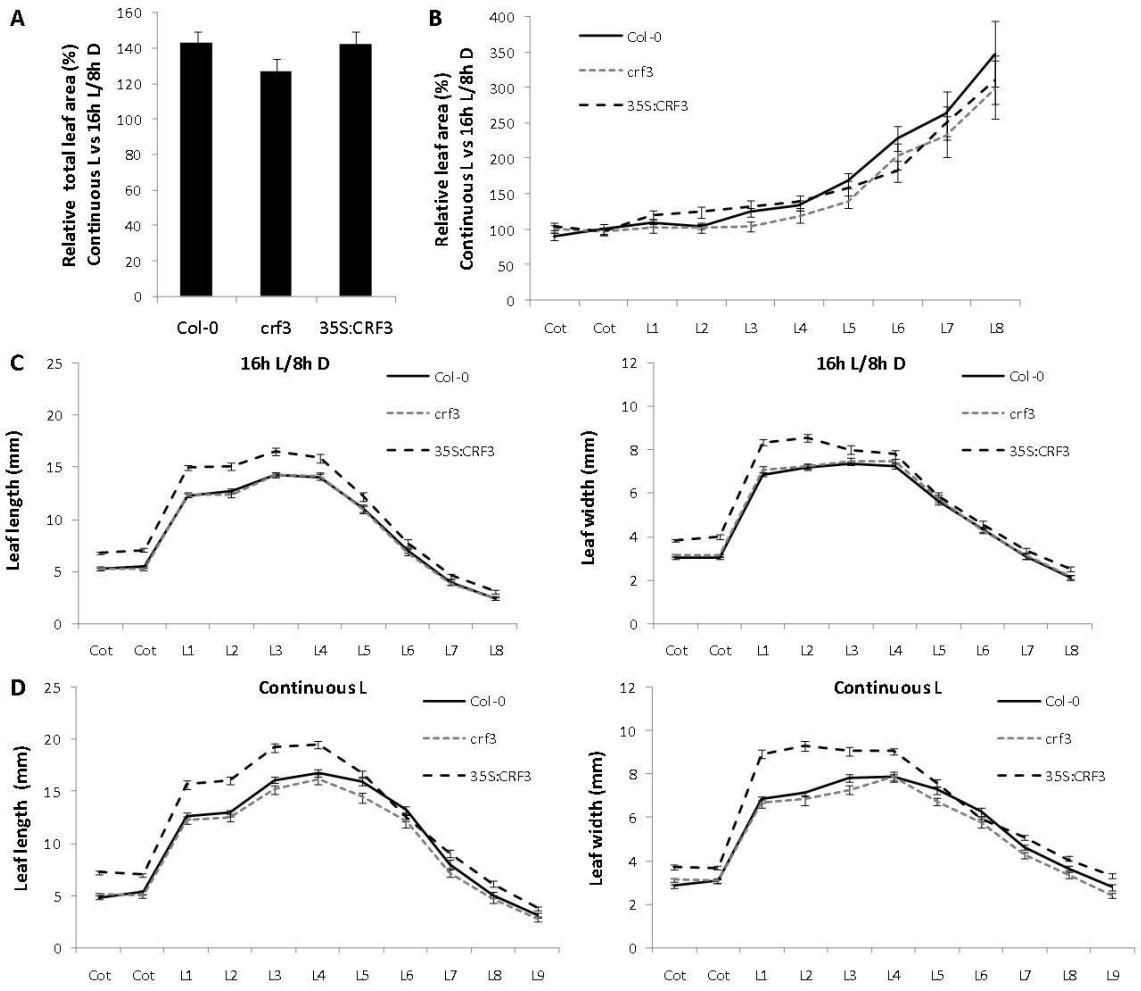
GUS staining

CRF3:GUS and *CYCB1;1:DB-GUS* plants were grown *in vitro* and harvested daily from 6 to 13 DAS. Subsequently, seedlings were incubated in heptane for 10 min and left to dry for 5 min. Then they were submersed in 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc) buffer [100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl, 50mM NaCl buffer (pH 7.0), 2mM $K_3[Fe(CN)_6]$, and 4mM X-Gluc], vacuum infiltrated for 10 min and incubated at 37°C for 8 h. Seedlings were cleared in 100% and 70% ethanol and kept in 90% lactic acid. Complete seedling or leaves 1&2 were mounted on slides and photographed under a stereomicroscope.

qRT-PCR

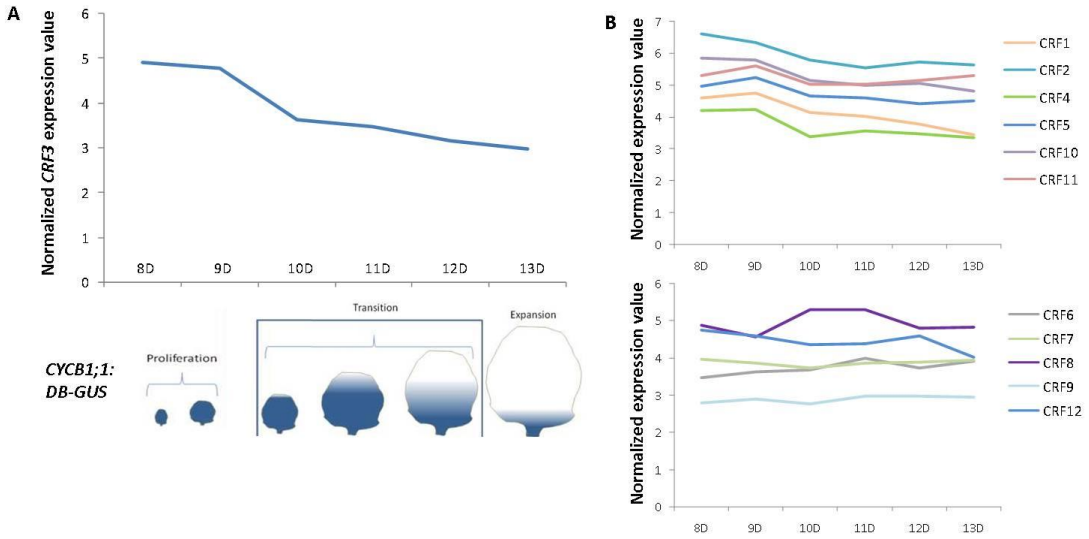
Complete seedlings were harvested in liquid nitrogen at 12 DAS. RNA was extracted according to a combined protocol of TRI Reagent RT (Molecular Research Center) and the RNeasy kit (QIAGEN) with on-column DNase (QIAGEN) digestion. cDNA was prepared from 500 ng to 1 µg of RNA with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. qRT-PCR was done on a LightCycler 480 (Roche Diagnostics) on 384-well plates with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's recommendations. All individual reactions were done in triplicate. Expression levels were normalized against the average of the housekeeping genes *CKA2*, and *CDKA1;1* and relative expression levels were determined with the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

SUPPLEMENTAL DATA



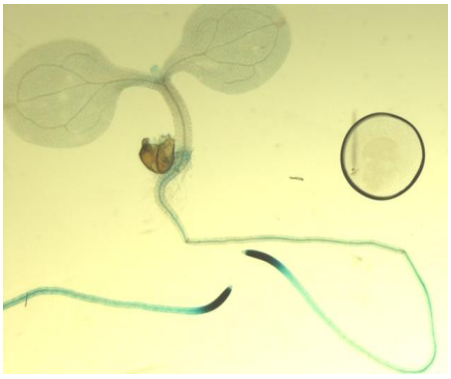
Supplemental Figure 1. Leaf area parameters of plants with modified *CRF3* levels in different growth conditions.

A, Relative increase in total rosette area of wild-type (Col-0), *crf3*, and *35S:CRF3* lines grown for 21 days in continuous light compared to long day conditions (16h light/8h dark). B, Relative increase in individual area of cotyledons (Cot) and leaves 1 to 8 (L1-L8) in continuous light versus long days for Col-0, *crf3* and *35S:CRF3*. C, Individual leaf length and leaf width, measured from leaf series made from 21-day-old plants grown in long day conditions. D, Individual leaf length and leaf width, measured from leaf series made from 21-day-old plants grown under continuous light. Error bars are SE (n = 16).



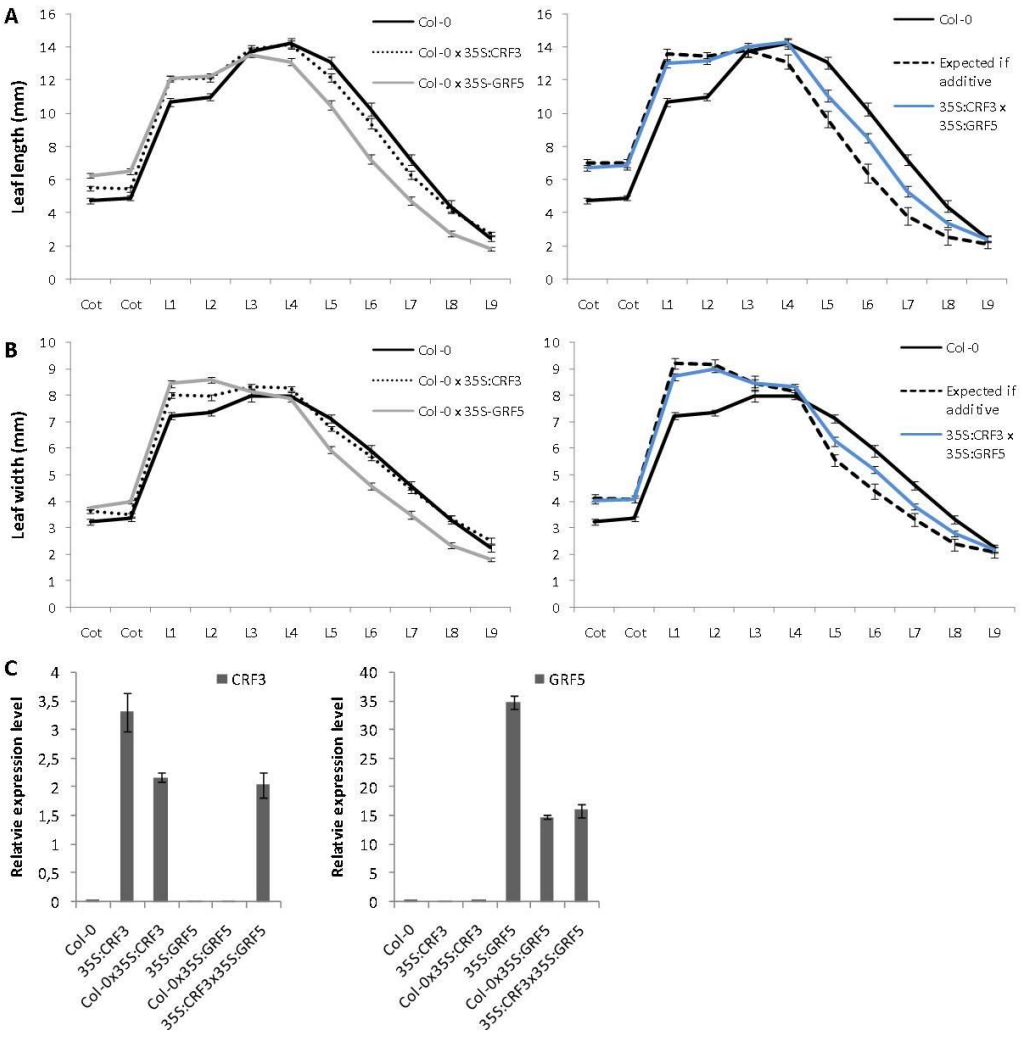
Supplemental Figure 2. *CRF* expression levels during leaf development.

A and B, Normalized expression values were calculated from AGRONOMICS1 tiling arrays by Andriankaja et al. (2012), during development of leaf 3. A, *CRF3* transcript level from days 8 to 13 during leaf 3 development, covering the subsequent phases of cell proliferation, transition and expansion, as indicated in the cartoon. Blue staining represents *CYCB1;1:DB-GUS* expression. B, Transcript levels of the other 11 CRFs.



Supplemental Figure 3. *CRF3* expression

CRF3:GUS-2 staining a seedling at 6 DAS.



Supplemental figure 4. Leaf parameters and transcript levels of *35S:CRF3* plants crossed with *35S:GRF5* plants.

A and B, Leaf length (A) and width (B) of cotyledons (Cot) and leaves 1 to 9 (L1-L9), measured from leaf series of wild-type (Col-0) plants, heterozygous parents and crossed plants at 21 DAS. Error bars are SE ($n \geq 14$). Values expected if additive were calculated as the sum of the single heterozygous lines minus the wild type. C, Relative *CRF3* and *GRF5* expression levels analyzed by qRT-PCR in 12-day-old shoots. Error bars are SE ($n \geq 2$).

REFERENCES

Andriankaja M, Dhondt S, De Bodt S, Vanhaeren H, Coppens F, De Milde L, Mühlenbock P, Skirycz A, Gonzalez N, Beemster GTS, Inzé D (2012) Exit from proliferation during leaf development in *Arabidopsis thaliana*: a not-so-gradual process. Developmental Cell **22:** 64-78

Argyros RD, Mathews DE, Chiang YH, Palmer CM, Thibault DM, Etheridge N, Argyros DA, Mason MG, Kieber JJ, Schaller GE (2008) Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. Plant Cell **20:** 2102-2116

Atta R, Laurens L, Boucheron-Dubuisson E, Guivarc'h A, Carnero E, Giraudat-Pautot V, Rech P, Chriqui D (2009) Pluripotency of Arabidopsis xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown in vitro. Plant Journal **57:** 626-644

Beemster GTS, De Veylder L, Vercruysse S, West G, Rombaut D, Van Hummelen P, Galichet A, Gruissem W, Inzé D, Vuylsteke M (2005) Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. Plant Physiology **138:** 734-743

Brandstatter I, Kieber JJ (1998) Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. Plant Cell **10:** 1009-1019

Braun N, Wyrzykowska J, Muller P, David K, Couch D, Perrot-Rechenmann C, Fleming AJ (2008) Conditional Repression of AUXIN BINDING PROTEIN1 Reveals That It Coordinates Cell Division and Cell Expansion during Postembryonic Shoot Development in Arabidopsis and Tobacco. Plant Cell **20:** 2746-2762

Brenner WG, Ramireddy E, Heyl A, Schmulling T (2012) Gene regulation by cytokinin in Arabidopsis. Front Plant Sci **3:** 8

Colón-Carmona A, You R, Haimovitch-Gal T, Doerner P (1999) Spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. Plant Journal **20:** 503-508

Cosgrove DJ (2005) Growth of the plant cell wall. Nature Reviews Molecular Cell Biology **6:** 850-861

Cutcliffe JW, Hellmann E, Heyl A, Rashotte AM (2011) CRFs form protein-protein interactions with each other and with members of the cytokinin signalling pathway in Arabidopsis via the CRF domain. J Exp Bot **62:** 4995-5002

D'Agostino IB, Deruere J, Kieber JJ (2000) Characterization of the response of the arabidopsis response regulator gene family to cytokinin. Plant Physiol **124:** 1706-1717

Dewitte W, Scofield S, Alcasabas AA, Maughan SC, Menges M, Braun N, Collins C, Nieuwland J, Prinsen E, Sundaresan V, Murray JAH (2007) *Arabidopsis* CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proceedings of the National Academy of Sciences of the United States of America **104:** 14537-14542

Donnelly PM, Bonetta D, Tsukaya H, Dengler RE, Dengler NG (1999) Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. Developmental Biology **215:** 407-419

Dortay H, Gruhn N, Pfeifer A, Schwerdtner M, Schmulling T, Heyl A (2008) Toward an interaction map of the two-component signaling pathway of Arabidopsis thaliana. Journal of Proteome Research **7:** 3649-3660

Dortay H, Mehnert N, Burkle L, Schmulling T, Heyl A (2006) Analysis of protein interactions within the cytokinin-signaling pathway of Arabidopsis thaliana. Febs Journal **273:** 4631-4644

Eloy NB, de Freitas Lima M, Van Damme D, Vanhaeren H, Gonzalez N, De Milde L, Hemerly AS, Beemster GTS, Inzé D, Ferreira PCG (2011) The APC/C *subunit 10* plays an essential role in cell proliferation during leaf development. Plant Journal **68:** 351-363

Gonzalez N, Beemster GTS, Inzé D (2009) David and Goliath: what can the tiny weed *Arabidopsis* teach us to improve biomass production in crops? Current Opinion in Plant Biology **12:** 157-164

Gonzalez N, De Bodt S, Sulpice R, Jikumaru Y, Chae E, Dhondt S, Van Daele T, De Milde L, Weigel D, Kamiya Y, Stitt M, Beemster GTS, Inzé D (2010) Increased leaf size: different means to an end. Plant Physiology **153:** 1261-1279

Gonzalez N, Vanhaeren H, Inzé D (2012) Leaf size control: complex coordination of cell division and expansion. Trends in Plant Science **in press**

Gu YQ, Wildermuth MC, Chakravarthy S, Loh YT, Yang CM, He XH, Han Y, Martin GB (2002) Tomato transcription factors Pti4, Pti5, and Pti6 activate defense responses when expressed in Arabidopsis. Plant Cell **14:** 817-831

Ham BK, Park JM, Lee SB, Kim MJ, Lee IJ, Kim KJ, Kwon CS, Paek KH (2006) Tobacco Tsip1, a DnaJ-type Zn finger protein, is recruited to and potentiates Tsi1-mediated transcriptional activation. Plant Cell **18:** 2005-2020

Holst K, Schmulling T, Werner T (2011) Enhanced cytokinin degradation in leaf primordia of transgenic Arabidopsis plants reduces leaf size and shoot organ primordia formation. Journal of Plant Physiology **168:** 1328-1334

Horiguchi G, Kim G-T, Tsukaya H (2005) The transcription factor AtGRF5 and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of *Arabidopsis thaliana*. Plant Journal **43:** 68-78

Horiguchi G, Nakayama H, Ishikawa N, Kubo M, Demura T, Fukuda H, Tsukaya H (2011) ANGUSTIFOLIA3 Plays Roles in Adaxial/Abaxial Patterning and Growth in Leaf Morphogenesis. Plant and Cell Physiology **52:** 112-124

Hutchison CE, Li J, Argueso C, Gonzalez M, Lee E, Lewis MW, Maxwell BB, Perdue TD, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2006) The Arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. Plant Cell **18:** 3073-3087

Hwang I, Sheen J (2001) Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature **413:** 383-389

Hwang I, Sheen J, Muller B (2012) Cytokinin signaling networks. Annu Rev Plant Biol **63:** 353-380

Imamura A, Hanaki N, Nakamura A, Suzuki T, Taniguchi M, Kiba T, Ueguchi C, Sugiyama T, Mizuno T (1999) Compilation and characterization of Arabidopsis thaliana response regulators implicated in His-Asp phosphorelay signal transduction. Plant and Cell Physiology **40:** 733-742

Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T (2001) Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature **409:** 1060-1063

Kazama T, Ichihashi Y, Murata S, Tsukaya H (2010) The mechanism of cell cycle arrest front progression explained by a *KLUH/CYP78A5*-dependent mobile growth factor in developing leaves of *Arabidopsis thaliana*. Plant and Cell Physiology **51:** 1046-1054

Kiba T, Yamada H, Sato S, Kato T, Tabata S, Yamashino T, Mizuno T (2003) The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in Arabidopsis thaliana. Plant and Cell Physiology **44:** 868-874

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **103:** 814-819

Krizek BA (2009) Making bigger plants: key regulators of final organ size. Current Opinion in Plant Biology **12:** 17-22

Lee BH, Ko J-H, Lee S, Lee Y, Pak J-H, Kim JH (2009) The Arabidopsis *GRF-INTERACTING FACTOR* gene family performs an overlapping function in determining organ size as well as multiple developmental properties. Plant Physiology **151:** 655-668

Lee DJ, Kim S, Ha YM, Kim J (2008) Phosphorylation of Arabidopsis response regulator 7 (ARR7) at the putative phospho-accepting site is required for ARR7 to act as a negative regulator of cytokinin signaling. Planta **227:** 577-587

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:** 402-408

Lohrmann J, Buchholz G, Keitel C, Sweere U, Kircher S, Baurle I, Kudla J, Schafer E, Harter K (1999) Differential expression and nuclear localization of response regulator-like proteins from Arabidopsis thaliana. Plant Biology **1:** 495-505

Mahonen AP, Bonke M, Kauppinen L, Riikonen M, Benfey PN, Helariutta Y (2000) A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. Genes & Development **14:** 2938-2943

Mason MG, Mathews DE, Argyros DA, Maxwell BB, Kieber JJ, Alonso JM, Ecker JR, Schaller GE (2005) Multiple type-B response regulators mediate cytokinin signal transduction in *Arabidopsis*. Plant Cell **17:** 3007-3018

Miyata S, Urao T, Yamaguchi-Shinozaki K, Shinozaki K (1998) Characterization of genes for two-component phosphorelay mediators with a single HPt domain in Arabidopsis thaliana. Febs Letters **437:** 11-14

Mok M (1994) Cytokinins and plant development: an overview. *In* D Mok, M Mok, eds, Cytokinins: chemistry, activity, and function, Boca Raton, FL: CRC, pp 155-166

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum **15:** 473-497

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. Plant Cell **16:** 1365-1377

Oh E, Kang H, Yamaguchi S, Park J, Lee D, Kamiya Y, Choi G (2009) Genome-wide analysis of genes targeted by PHYTOCHROME INTERACTING FACTOR 3-LIKE5 during seed germination in *Arabidopsis*. Plant Cell **21:** 403-419

Oh E, Kim J, Park E, Kim J-I, Kang C, Choi G (2004) PIL5, a phytochrome-interacting basic helix-loop-helix protein, is a key negative regulator of seed germination in *Arabidopsis thaliana*. Plant Cell **16:** 3045-3058

Oh E, Yamaguchi S, Hu J, Yusuke J, Jung B, Paik I, Lee H-S, Sun T-p, Kamiya Y, Choi G (2007) PIL5, a phytochrome-interacting bHLH protein, regulates gibberellin responsiveness by binding directly to the *GAI* and *RGA* promoters in *Arabidopsis* seeds. Plant Cell **19:** 1192-1208

Oh E, Yamaguchi S, Kamiya Y, Bae G, Chung W-I, Choi G (2006) Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in Arabidopsis. Plant Journal **47:** 124-139

Okazaki K, Kabeya Y, Suzuki K, Mori T, Ichikawa T, Matsui M, Nakanishi H, Miyagishima S (2009) The PLASTID DIVISION1 and 2 Components of the Chloroplast Division Machinery Determine the Rate of Chloroplast Division in Land Plant Cell Differentiation. Plant Cell **21:** 1769-1780

Park JM, Park CJ, Lee SB, Ham BK, Shin R, Paek KH (2001) Overexpression of the tobacco Tsi1 gene encoding an EREBP/AP2-Type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. Plant Cell **13:** 1035-1046

Rashotte AM, Goertzen LR (2010) The CRF domain defines Cytokinin Response Factor proteins in plants. Bmc Plant Biology **10**

Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2006) A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proceedings of the National Academy of Sciences of the United States of America **103:** 11081-11085

Riefler M, Novak O, Strnad M, Schmulling T (2006) Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18:** 40-54

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH (1999) Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science **283:** 1541-1544

Sakai H, Aoyama T, Bono H, Oka A (1998) Two-component response regulators from Arabidopsis thaliana contain a putative DNA-binding motif. Plant and Cell Physiology **39:** 1232-1239

Scarpella E, Barkoulas M, Tsiantis M (2010) Control of Leaf and Vein Development by Auxin. Cold Spring Harbor Perspectives in Biology **2**

Schlereth A, Möller B, Liu W, Kientz M, Flipse J, Rademacher EH, Schmid M, Jürgens G, Weijers D (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. Nature **464:** 913-916

Shi XL, Gupta S, Rashotte AM (2012) Solanum lycopersicum cytokinin response factor (SlCRF) genes: characterization of CRF domain-containing ERF genes in tomato. J Exp Bot **63:** 973-982

Su YH, Liu YB, Zhang XS (2011) Auxin-Cytokinin Interaction Regulates Meristem Development. Mol Plant **4:** 616-625

Sugimoto K, Jiao YL, Meyerowitz EM (2010) Arabidopsis Regeneration from Multiple Tissues Occurs via a Root Development Pathway. Developmental Cell **18:** 463-471

Suzuki T, Imamura A, Ueguchi C, Mizuno T (1998) Histidine-containing phosphotransfer (HPt) signal transducers implicated in His-to-Asp phosphorelay in Arabidopsis. Plant and Cell Physiology **39:** 1258-1268

Suzuki T, Miwa K, Ishikawa K, Yamada H, Aiba H, Mizuno T (2001a) The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. Plant and Cell Physiology **42:** 107-113

Suzuki T, Sakurai K, Imamura A, Nakamura A, Ueguchi C, Mizuno T (2000) Compilation and characterization of histidine-containing phosphotransmitters implicated in His-to-Asp phosphorelay in plants: AHP signal transducers of Arabidopsis thaliana. Bioscience Biotechnology and Biochemistry **64:** 2486-2489

Suzuki T, Sakurai K, Ueguchi C, Mizuno T (2001b) Two types of putative nuclear factors that physically interact with histidine-containing phosphotransfer (Hpt) domains, signaling mediators in His-to-Asp phosphorelay, in Arabidopsis thaliana. Plant and Cell Physiology **42:** 37-45

Taniguchi M, Sasaki N, Tsuge T, Aoyama T, Oka A (2007) ARR1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions. Plant and Cell Physiology **48:** 263-277

To JPC, Haberer G, Ferreira FJ, Deruere J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2004) Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. Plant Cell **16:** 658-671

Ueguchi C, Koizumi H, Suzuki T, Mizuno T (2001) Novel family of sensor histidine kinase genes in Arabidopsis thaliana. Plant and Cell Physiology **42:** 231-235

Vanneste S, De Rybel B, Beemster GTS, Ljung K, De Smet I, Van Isterdael G, Naudts M, Iida R, Gruissem W, Tasaka M, Inzé D, Fukaki H, Beeckman T (2005) Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in *Arabidopsis thaliana*. Plant Cell **17:** 3035-3050

Vercruyssen L, Gonzalez N, Werner T, Schmulling T, Inze D (2011) Combining Enhanced Root and Shoot Growth Reveals Cross Talk between Pathways That Control Plant Organ Size in Arabidopsis. Plant Physiol **155:** 1339-1352

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmulling T (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15:** 2532-2550

Xu K, Liu J, Fan M, Xin W, Hu Y, Xu C (2012) A genome-wide transcriptome profiling reveals the early molecular events during callus initiation in Arabidopsis multiple organs. Genomics

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant and Cell Physiology **42:** 1017-1023

Zhou JM, Tang XY, Martin GB (1997) The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. Embo Journal **16:** 3207-3218

6

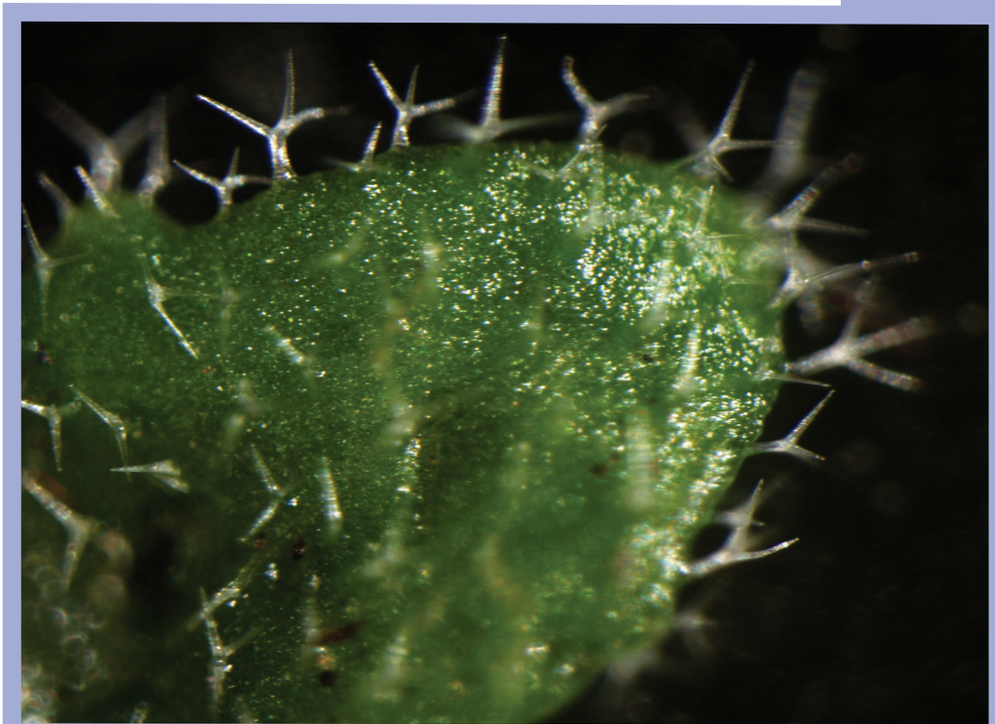


Image: Close-up view of an Arabidopsis Col-0 rosette leaf.

Chapter 6: Concluding remarks and future perspectives

CYTOKININS AS A COMMON THEME

Phytohormones integrate environmental cues with endogenous signals resulting in an adequate growth response in diverse situations thus providing the plant with the developmental plasticity needed for survival. Cytokinins are well known to negatively affect root growth, while promoting shoot growth. By ectopically expressing *CYTOKININ OXIDASE/DEHYDROGENASE 3* (*CKX3*) from a root-specific promoter, cytokinin deficiency is confined to the root, resulting in increased root growth, simultaneously limiting the inhibiting effect on shoot growth. The combination of overexpression of *CKX3* with functionally distinct transgenes enhancing shoot growth uncovered synergistic effects, allowing the dissection of the genetic interaction of the hormone response pathways involved. As such, we propose that cytokinins attenuate the brassinosteroid response during leaf growth and lateral root initiation and elongation, and the effect on lateral root growth was corroborated by measurements of the *CKX3* overexpressing root system grown on medium supplemented with brassinolide (BL). In addition, we hypothesize that cytokinins antagonize gibberellins during leaf development, in agreement with other reports in literature. Future work should aim to identify the molecular integrators of the cross talk between cytokinins, brassinosteroids and gibberellins.

Enhanced cytokinin breakdown also reduced the increased leaf growth resulting from overexpression of *GROWTH REGULATING FACTOR 5* (*GRF5*), revealing that GRF5 and cytokinins are likely to work together to stimulate leaf growth. By analyzing the expression of the *CYCB1;1:DB-GUS* mitotic marker in young leaves overexpressing *GRF5* and/or treated with a synthetic cytokinin, 6-benzylaminopurine (BAP), we have sought to elucidate the cellular nature of the interaction. This revealed that both GRF5 and cytokinins likely promote the rate of leaf cell proliferation as well as the duration of the cell proliferation phase, and that they synergistically stimulate these processes (Fig. 1, blue arrows). It seems rather unlikely that *GRF5* directly regulates *CYCB1;1* expression or cytokinin metabolism and there is no evidence for the regulation of *GRF5* transcript

levels by cytokinins. Moreover, combined overexpression of *GRF5* with *CYTOKININ RESPONSE FACTOR 3* (*CRF3*), yielded additive increases in leaf area, suggesting that they work independently and that GRF5 action does not impinge on the branch of the cytokinin signaling pathway downstream of CRF3 (Fig. 1, blue arrows). Therefore, the actual point(s) of cross talk between GRF5 signaling and the cytokinin response pathway to cooperatively stimulate cell proliferation in a developing leaf remain(s) to be identified.

Although we showed that overexpression of *CRF3* increases cell number in Arabidopsis leaves without affecting cell size, *CRF3* overexpression does not seem to prolong the cell proliferation phase, in contrast to *GRF5* overexpression and cytokinin treatment. The endoreduplication index rather suggests that overexpression of *CRF3* fastens leaf development so that the increased leaf size originates from an increase in the cell proliferation rate. Since *CRF3*, like *CRF2*, can be transcriptionally induced by auxin (Vanneste et al., 2005; Schlereth et al., 2010), the faster leaf development hints at the potential involvement of auxin in CRF3-stimulated leaf growth, which deserves further investigation. Also, a detailed cellular kinematic analysis and crossing with the *CYCB1;1:DB-GUS* marker line should unequivocally determine the cellular nature of the *CRF3* overexpression phenotype. On top, transcript profiling of young leaves that are dividing and that start the transition from cell proliferation to cell expansion, coinciding with the highest expression of *CRF3*, should shed more light on the downstream effectors.

A similar experimental strategy, microarray analysis of transitioning leaves after induction of ANGUSTIFOLIA 3 (AN3) activity, was used to determine its downstream responses and direct transcriptional targets. AN3 also promotes the rate of leaf cell division and the duration of the cell proliferation phase, as determined by *CYCB1;1:DB-GUS* staining, and consistently AN3 promotes transcription favoring cell proliferation including *GRF5* transcription that is upregulated within two hours after AN3 induction (Fig. 1, black arrows). Moreover, *GRF6* and *GRF3* could be identified as putative direct targets of AN3, and since AN3 interacts with the GRFs, we hypothesize that the AN3/GRF transcriptional coactivator/ transcription factor complexes regulate *AN3* and *GRF* expression. Furthermore, *HOMEOBOX 33* (*HB33*), *HECATE1* (*HEC1*), *CRF2* and

CONSTANS-LIKE 5 (*COL5*) are rapidly induced after AN3 activation and genetic dissection of the AN3/GRF5 interaction and concomitant expression analysis of these downstream target genes leads us to propose the following model: AN3/GRF5 modulates transcription of a subset of genes including *HB33* and *HEC1*, while AN3 interacts with other GRFs, most likely *GRF6* and *GRF3*, or with other transcription factors to regulate expression of other target genes like *CRF2* and *COL5*. In addition, GRF5 could control transcription independently from AN3 (Fig. 1, black arrows). Promoter-luciferase activation assays complemented with Chromatin immunoprecipitation (ChIP) experiments and transcript profiling after induction of GRF functions should help verifying this model in practice.

Remarkably, CRF2 has been described to stimulate chloroplast division (Okazaki et al., 2009) and we report that *GRF5* overexpression also enhances chloroplast division. Therefore it is tempting to speculate that GRF5, in complex with AN3, does stimulate *CRF2* expression to promote chloroplast division (Fig. 1, black arrows). In that regard it is interestingly to note that the *altered meristem program 1* (*amp1*) mutant has a higher cytokinin content and *CRF2* expression (Griffiths et al., 2011), while *AMP1* is differentially expressed in *GRF5* overexpressing seedlings. However, *AN3* overexpressing plants are not darker green and *CRF2* is not differentially expressed when *GRF5* is overexpressed, suggesting that GRF5 promotes chloroplast division independently of AN3 and CRF2 (Fig. 1, purple arrows). The lack of cross talk with CRF2 goes hand in hand with the independent mode of action of GRF5 and CRF3 and is corroborated by the finding that all three genes are directly repressed in parallel by PHYTOCHROME INTERACTING FACTOR 1 (PIF1) during seed germination in the dark (Oh et al., 2009). An interesting note, also *HEC1* is directly repressed by PIF1 (Oh et al., 2009), indicating that the same transcription factors are coordinately regulated during different processes.

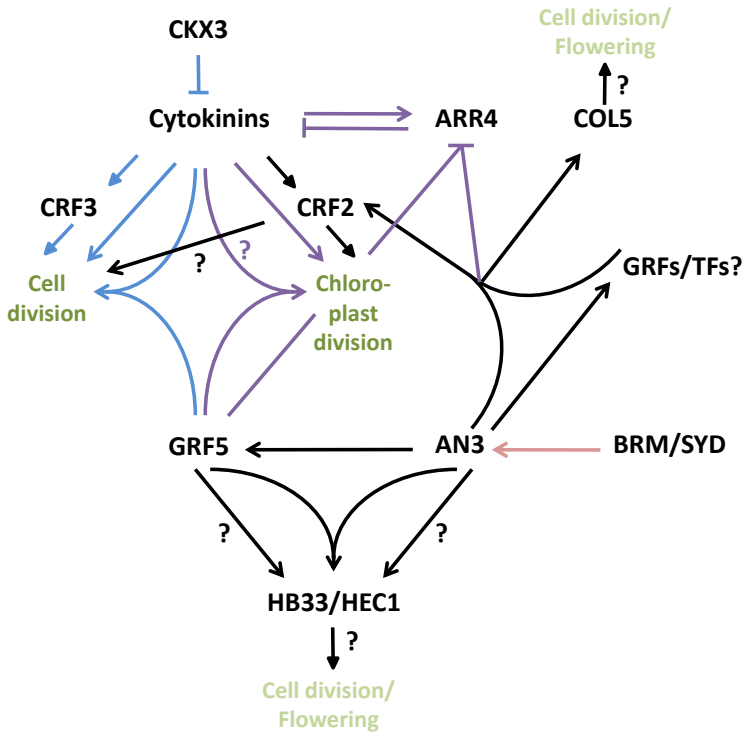


Figure 1. Model integrating the molecular players identified and/or characterized during the course of the PhD research.

Blue arrows: cytokinins work together with GRF5 to promote cell division during leaf development. CKX3 breaks down cytokinins (Werner et al., 2003). CRF3 is part of the cytokinin response pathway (Rashotte et al., 2006) and shown to stimulate leaf cell division, most likely independently of GRF5. Black arrows: AN3 activates transcription of *GRF5* and they are proposed to regulate *HB33* and *HEC1* transcription together as a complex, although independent regulation cannot be excluded (question marks). In addition, AN3 activates transcription of and interacts with other GRFs or other transcription factors (TFs), to upregulate *COL5* and *CRF2* expression. CRF2 is part of the cytokinin response pathway (Rashotte et al., 2006) and stimulates chloroplast division (Okazaki et al., 2009). The involvement of CRF2, HB33, HEC1 and COL5 during leaf cell division and/or flower development needs further investigation. Purple arrows: GRF5 stimulates chloroplast division, similarly to cytokinins, but the question remains if GRF5 cooperates with cytokinins to stimulate chloroplast division. GRF5 and AN3 negatively regulate *ARR4* expression, which is suggested to reinforce cytokinin signaling, since ARR4 negatively feeds back on the cytokinin response pathway (To et al., 2004). Red arrow: AN3 associates with SWI/SNF chromatin remodeling complexes around the ATPases BRM and SYD. Question marks indicate speculative regulations.

Nevertheless, it will be of great interest to test if AN3, like GRF5, displays synergism with cytokinins to promote leaf cell division. Besides the induction of *CRF2* by AN3, AN3 and most likely GRF5 negatively regulate expression of A-type *ARABIDOPSIS RESPONSE REGULATOR 4* (*ARR4*), possibly reinforcing cytokinin signaling in the proliferation zone (Fig. 1, purple arrows). BAP treatment of leaves co-expressing *AN3* and *CYCB1;1-DB-GUS* provides a straightforward way to investigate the relationship between AN3 and cytokinins. Furthermore, the question if GRF5 also cooperates with cytokinins to stimulate chloroplast division remains to be answered (Fig. 1, purple arrows). Of interest would be to test the sensitivity of *GRF5* overexpressing plants towards exogenous cytokinins during the regeneration of green shoot tissue from callus and in chlorophyll retention upon dark-induced senescence. The analysis of inducible *GRF5* overexpressing lines will also gain more insight.

Sequence homology of AN3 with human SYNOVIAL TRANSLOCATION (SYT), a protein that interacts with human BRAHMA (BRM) and BRAHMA RELATED GENE (BRG1) chromatin remodeling ATPases (Thaete et al., 1999; Nagai et al., 2001; Perani et al., 2003), hinted at the involvement of chromatin modifications in AN3-mediated regulation of transcription, but evidence was still missing. Moreover, although knowledge starts to accumulate on core components of plant SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin remodeling complexes, very few associated proteins have been identified in Arabidopsis. Here, we show that AN3 is part of SWI/SNF chromatin remodeling complexes around BRM and SYD, and hypothesize that AN3 functions as an interacting protein that guides the complexes towards the DNA to regulate target gene transcription (Fig. 1, red arrow). In agreement, *GRF5*, *HB33*, *HEC1*, *CRF2*, *COL5* as well as *AN3* transcript levels were altered in *brm* shoots, and in addition the presence of BRM could be confirmed at the *HEC1* promoter in leaves. It is not known however with which particular SWI/SNF subunit AN3 interacts and yeast two-hybrid analyses are underway to identify the direct interaction partners. SWI/SNF chromatin remodeling is of key importance to regulate developmental switches balancing cellular self-renewal with cell differentiation, both by repression and activation of transcription. Since AN3, GRF5, HEC1 and COL5 were reported to be involved in flowering (Kim et al., 2003; Gremski et al., 2007; Hassidim et al., 2009; Lee et al., 2009; Crawford and

Yanofsky, 2011), their potential association with BRM and SYD in the regulation of transcription during flowering presents an interesting topic for future research.

In conclusion, this work contributes to a deeper understanding of the role of cytokinins in promoting leaf cell proliferation during Arabidopsis development. Cytokinins somehow link the majority of the genes that were characterized in more detail during the course of this PhD thesis, but more research should be carried out to pinpoint the molecular players that integrate the diverse signaling pathways.

CANDIDATE GENES FOR IMPROVING PLANT PRODUCTIVITY

Through translational research, the findings in Arabidopsis can be applied to crop species, which is illustrated for example by the overexpression of Arabidopsis *GIBBERELLIN 20-OXIDASE 1* (*GA20ox1*) in maize that results in a 40% increase in leaf elongation rate due to an enlargement of the cell division zone (Nelissen et al., 2012). In crops, combining different beneficial phenotypic traits by stacking multiple transgenes has gained much interest. It is shown here that simultaneous overexpression of two genes that enhance growth is a powerful strategy to further increase growth of Arabidopsis. As such, the simultaneous overexpression of *GRF5* and *AN3* or *GRF5* and *CRF3* results in larger leaves whose increases in area correspond to the sum of the growth increases of the single overexpressing lines. Also enhanced root growth could be combined with enhanced shoot growth, resulting in an increase in overall plant growth. Moreover, the effects on lateral root growth and/or leaf size were more than additive when ectopic *CKX3* expression was combined with *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) or *GA20ox1* overexpression, demonstrating that this experimental strategy has unexpected additional advantages.

Overexpression of *GRF5* not only promotes cell division during leaf development, but also enhances chloroplast division, photosynthetic capacity and tolerance to nitrogen deprivation. Because chloroplasts serve as major energy source for the plant, altering photosynthetic efficiency has become an important aspect in the genetic improvement of crop productivity. Likewise, improving nitrogen use efficiency has become important, due to the high cost of nitrogen fertilizers for society and the environment. Therefore, it

will be of particular interest to test whether plants co-expressing *GRF5* with other transgenes are able to maintain the increased photosynthetic capacity and the ability to grow better in nitrogen-depleted conditions. Furthermore, root-specific ectopic expression of *CKX* leads to an increased accumulation of minerals in the leaves, enhanced chlorophyll retention under nutrient-limiting conditions and better survival under severe drought conditions (Werner et al., 2010). Thus, enhanced root growth enables better exploitation of soil nutrients and water that could be used to sustain increased shoot growth. Simultaneous enhancement of the photosynthetic capacity could synergistically improve productivity and all together provide the plant with a tremendous advantage in adverse environmental conditions. Therefore, it would be worthwhile to test the growth response of the here presented double overexpressing lines to diverse abiotic stress conditions.

The genes described in this thesis enhance Arabidopsis leaf growth when overexpressed predominantly by increasing leaf cell proliferation, specifically by affecting the rate of cell division and/or the duration of the cell proliferation phase. This creates the opportunity to combine these genes with genes that promote other processes like cell expansion or meristemoid division to further improve leaf growth.

It becomes clear that there are infinite possibilities to stack genes with great potential to improve productivity. An additional beneficial trait for instance of *CKX* and *GRF5* overexpressing plants is their increase in seed size (Van Daele et al., 2012). However, total seed number is decreased, but this could potentially be compensated for by the combination with genes enhancing seed number, ultimately resulting in more seed yield. An increased cytokinin content in the inflorescence of the *ckx3ckx5* double mutant for example led to a 55% increase in seed yield (Bartrina et al., 2011). Furthermore, the core components of SWI/SNF chromatin remodeling complexes were shown to regulate virtually every aspect of plant development, and we showed that manipulation of *BRM* and *SWI3C* expression levels has the ability to increase leaf growth. Future research should aim to explore the feasibility of combining ectopic expression of more genes and mutations in genes encoding proteins with distinct functions.

It will be very promising to study the effects of the above described transgenes in crops, individually and stacked, and to analyze growth in field conditions. In that respect, maize plants overexpressing *AN3* and *GRF5* are currently under investigation in our research group and field trials with maize plants overexpressing *GA20ox1* are ongoing.

REFERENCES

Bartrina I, Otto E, Strnad M, Werner T, Schmulling T (2011) Cytokinin Regulates the Activity of Reproductive Meristems, Flower Organ Size, Ovule Formation, and Thus Seed Yield in Arabidopsis thaliana. Plant Cell **23:** 69-80

Crawford BCW, Yanofsky MF (2011) HALF FILLED promotes reproductive tract development and fertilization efficiency in Arabidopsis thaliana. Development **138:** 2999-3009

Gremski K, Ditta G, Yanofsky MF (2007) The HECATE genes regulate female reproductive tract development in Arabidopsis thaliana. Development **134:** 3593-3601

Griffiths J, Barrero JM, Taylor J, Helliwell CA, Gubler F (2011) ALTERED MERISTEM PROGRAM 1 Is involved in Development of Seed Dormancy in Arabidopsis. Plos One **6**

Hassidim M, Harir Y, Yakir E, Kron I, Green RM (2009) Over-expression of CONSTANS-LIKE 5 can induce flowering in short-day grown Arabidopsis. Planta **230:** 481-491

Kim JH, Choi D, Kende H (2003) The AtGRF family of putative transcription factors is involved in leaf and cotyledon growth in *Arabidopsis*. Plant Journal **36:** 94-104

Lee BH, Ko J-H, Lee S, Lee Y, Pak J-H, Kim JH (2009) The Arabidopsis *GRF-INTERACTING FACTOR* gene family performs an overlapping function in determining organ size as well as multiple developmental properties. Plant Physiology **151:** 655-668

Nagai M, Tanaka S, Tsuda M, Endo S, Kato H, Sonobe H, Minami A, Hiraga H, Nishihara H, Sawa H, Nagashima K (2001) Analysis of transforming activity of human synovial sarcoma-associated chimeric protein SYT-SSX1 bound to chromatin remodeling factor hBRM/hSNF2 alpha. Proceedings of the National Academy of Sciences of the United States of America **98:** 3843-3848

Nelissen H, Rymen B, Jikumaru Y, Demuynck K, Van Lijsebettens M, Kamiya Y, Inze D, Beemster GT (2012) A local maximum in gibberellin levels regulates maize leaf growth by spatial control of cell division. Current Biology **22:** 1183-1187

Oh E, Kang H, Yamaguchi S, Park J, Lee D, Kamiya Y, Choi G (2009) Genome-wide analysis of genes targeted by PHYTOCHROME INTERACTING FACTOR 3-LIKE5 during seed germination in *Arabidopsis*. Plant Cell **21:** 403-419

Okazaki K, Kabeya Y, Suzuki K, Mori T, Ichikawa T, Matsui M, Nakanishi H, Miyagishima S (2009) The PLASTID DIVISION1 and 2 Components of the Chloroplast Division Machinery Determine the Rate of Chloroplast Division in Land Plant Cell Differentiation. Plant Cell **21:** 1769-1780

Perani M, Ingram CJE, Cooper CS, Garrett MD, Goodwin GH (2003) Conserved SNH domain of the proto-oncoprotein SYT interacts with components of the human chromatin remodelling complexes, while the QPGY repeat domain forms homo-oligomers. Oncogene **22:** 8156-8167

Rashotte AM, Mason MG, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2006) A subset of *Arabidopsis* AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proceedings of the National Academy of Sciences of the United States of America **103:** 11081-11085

Schlereth A, Möller B, Liu W, Kientz M, Flipse J, Rademacher EH, Schmid M, Jürgens G, Weijers D (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. Nature **464:** 913-916

Thaete C, Brett D, Monaghan P, Whitehouse S, Rennie G, Rayner E, Cooper CS, Goodwin G (1999) Functional domains of the SYT and SYT-SSX synovial sarcoma translocation proteins and co-localization with the SNF protein BRM in the nucleus. Human Molecular Genetics **8:** 585-591

To JPC, Haberer G, Ferreira FJ, Deruere J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2004) Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. Plant Cell **16:** 658-671

Van Daele I, Gonzalez N, Vercauteren I, de Smet L, Inze D, Roldan-Ruiz I, Vuylsteke M (2012) A comparative study of seed yield parameters in Arabidopsis thaliana mutants and transgenics. Plant Biotechnol J **10:** 488-500

Vanneste S, De Rybel B, Beemster GTS, Ljung K, De Smet I, Van Isterdael G, Naudts M, Iida R, Gruissem W, Tasaka M, Inzé D, Fukaki H, Beeckman T (2005) Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in *Arabidopsis thaliana*. Plant Cell **17:** 3035-3050

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmulling T (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15:** 2532-2550

Werner T, Nehnevajova E, Kollmer I, Novak O, Strnad M, Kramer U, Schmulling T (2010) Root-Specific Reduction of Cytokinin Causes Enhanced Root Growth, Drought Tolerance, and Leaf Mineral Enrichment in Arabidopsis and Tobacco. Plant Cell **22:** 3905-3920

The final size of plant organs is controlled by a complex regulatory network of molecular players governed by different plant hormones. By analyzing Arabidopsis plants showing enhanced growth due to overexpression of distinct genes at the morphological and molecular level, we have uncovered new genes and new interactions between existing genes thereby extending the regulatory network that controls plant growth.

First, when overexpression of *CKX3* and *BRI1* were combined, leaf growth and lateral root initiation and elongation were synergistically enhanced, suggesting that cytokinins attenuate the brassinosteroid response during leaf and lateral root development. Likewise, co-overexpression of *CKX3* with *GA20ox1* led to a synergistic increase in leaf size, indicating an antagonistic control of leaf growth by cytokinins and gibberellins.

Subsequently the transcriptional coactivator AN3 and the transcription factor GRF5, previously proposed to physically interact in order to stimulate cell division during leaf growth, were characterized in detail. Mitotic *CYCB1;1-DB-GUS* marker gene expression revealed that *AN3* and *GRF5* overexpression promote the rate of leaf cell proliferation as well as the duration of the cell proliferation phase. We showed that cytokinin treatment enhances the rate and duration of leaf cell proliferation similarly to *GRF5* overexpression. Moreover, they synergistically stimulate these processes. In addition, overexpression of *GRF5* stimulates chloroplast division, resulting most likely in a higher photosynthetic capacity and increased tolerance to nitrogen deprivation, traits that are also affected by cytokinins.

Next, induction of AN3 activity allowed the identification of *GRF5*, *GRF6*, and *GRF3* as putative direct target genes, while AN3 interacts with the GRFs, suggesting that AN3 and GRFs together regulate their own transcription. Additional transcriptional regulators, including *HB33*, *HEC1*, *CRF2*, and *COL5* were rapidly induced after AN3 activation and hypothesized to act directly downstream of AN3 and/or GRF5 to regulate leaf development. Furthermore, we provided evidence that AN3 associates with chromatin remodeling SWI/SNF complexes formed around the ATPases BRM or SYD and postulate that AN3 recruits chromatin modifying activity for efficient transcription of the above mentioned target genes.

Finally, we identified CRF3 as a new growth-promoting transcription factor and revealed that its overexpression increases leaf cell proliferation, rather than cell expansion, two

main cellular processes that contribute to the determination of final leaf size. Analysis of leaf areas of plants simultaneously overexpressing *CRF3* and *GRF5* however hinted at an independent mode of action.

All the above described genes are potential valuable candidates for genetic engineering to improve biomass production in crop species. In addition, particular genes that are often members of hormone response pathways might signal to integrate external cues, such as nitrogen availability and light, with internal growth requirements, rendering the plants more resistant to adverse environmental conditions, together ultimately benefitting life on earth.

De finale afmeting van plantenorganen wordt gecontroleerd door een complex netwerk van moleculaire spelers dat aangestuurd wordt door de verschillende plantenhormonen. Via de analyse zowel op morfologisch als moleculair niveau van Arabidopsis planten die een toename in groei vertonen door overexpressie van diverse genen, hebben we nieuwe genen en nieuwe interacties tussen gekende genen ontdekt, met als resultaat de uitbreiding van het netwerk dat plantengroei controleert.

Als eerste werd een synergistische toename in bladgroei en in de initiatie en elongatie van zijwortels geobserveerd, wanneer overexpressie van *CKX3* en *BRI1* werden gecombineerd. Dit suggereert dat cytokinines de respons op brassinosteroiden afzwakken tijdens de ontwikkeling van bladeren en zijwortels. Co-overexpressie van *CKX3* en *GA20ox1* leidde evenzo tot een synergistische toename in bladgrootte, wat wijst op een antagonistische controle van bladgroei door cytokinines en gibberellines.

Vervolgens werd een gedetailleerde karakterisatie uitgevoerd van de transcriptionele coactivator AN3 en de transcriptiefactor GRF5, die verondersteld worden een fysieke interactie aan te gaan om samen celdeling in groeiende bladeren te stimuleren. Het expressiepatroon van het *CYCB1;1-DB-GUS* merkergen onthulde dat overexpressie van *AN3* en *GRF5* zowel de snelheid van celdeling als de duur van de celdelingsfase stimuleert. Er werd tevens aangetoond dat behandeling met cytokininen, net zoals overexpressie van *GRF5*, de snelheid en duur van celdeling bevordert. Bovendien stimuleren ze deze processen op een synergistische wijze. GRF5 bevordert ook chloroplastdeling, hetgeen zeer waarschijnlijk resulteert in een verhoogde capaciteit voor fotosynthese en tolerantie voor stikstofgebrek, eigenschappen die ook door cytokininen beïnvloed worden.

Inductie van de activiteit van AN3 liet ons vervolgens toe om *GRF5*, *GRF6* en *GRF3* te identificeren als vermoedelijke doelgenen, zogenaamde target genen. Dit suggereert, gezien de fysieke binding van AN3 met de GRFs, dat ze samen hun eigen transcriptie reguleren. Andere transcriptieregulatoren waaronder *HB33*, *HEC1*, *CRF2* en *COL5* werden snel geïnduceerd na activatie van AN3 en daardoor verondersteld om direct downstream van AN3 en/of GRF5 te functioneren tijdens bladgroei. Daarenboven bewezen we dat AN3 een associatie vormt met SWI/SNF chromatine ‘remodeling’ complexen die gevormd worden rond de ‘ATPasen’ BRM of SYD en postuleren we dat

AN3 de chromatine ‘remodeling’ activiteit aanbrengt voor een efficiënte transcriptie van bovenvermelde target genen.

Ten slotte identificeerden we CRF3 als een nieuwe transcriptiefactor die groei stimuleert en toonden we aan dat overexpressie van *CRF3* celdeling bevordert in plaats van celexpansie, de twee voornaamste cellulaire processen die bijdragen tot de uiteindelijke bladgrootte. De analyse van de bladoppervlakte van planten die tegelijkertijd *CRF3* en *GRF5* tot overexpressie brengen, doet een onafhankelijke werkwijze vermoeden.

Alle hierboven beschreven genen zijn mogelijke waardevolle kandidaten die de biomassaproductie van gewassen kunnen verbeteren door genetische manipulatie. Bovendien kunnen bepaalde genen die doorgaans functioneren in signalisatiewegen van hormonen, externe elementen zoals de beschikbaarheid van stikstof en licht, koppelen aan de interne behoeften, resulterend in een betere resistentie van de planten tegen nadelige omgevingscondities om zo uiteindelijk het leven op aarde ten goede te komen.

Graag wil ik iedereen van harte bedanken die heeft bijgedragen niet alleen tot deze doctoraatsthesis, maar ook tot de weg die ik heb afgelegd om dit te verwezenlijken.

Via mijn masterthesis kwam ik terecht in het ‘Plant Systems Biology’ departement en na een leerrijk jaar in een toffe groep was ik gebeten door de ‘Plant-Microbe’. Hoewel ik er tijdens mijn studies niet had bij stilgestaan, werd ik aangemoedigd om een doctoraat te starten. Daarom wil ik Marcelle Holsters en Sofie Goormachtig bedanken voor de kans die ze me gegeven hebben door in mij te geloven als doctoraatsstudente.

Een heel fijn jaar en twee iets minder succesvolle beursaanvragen later, liet ik de wereld van de vlinderbloemigen en symbiotische stikstoffixatie achter me en werd ik ondergedompeld in ‘Systems Biology of Yield’ van Arabidopsis. Door het laaiend enthousiasme van Dirk Inzé is de vraag “wat bepaalt de uiteindelijke grootte van plantenorganen?” een vraag die nu ook mij uitermate fascineert. Ik wil Dirk in het bijzonder bedanken voor het vertrouwen dat hij in mijn gesteld heeft, de zeer professionele begeleiding en wetenschappelijke toewijding. Dat het evenzeer belangrijk is om te beslissen wat niet te doen, is een van de vele dingen die ik heb bijgeleerd, ook al ging dat soms gepaard met de nodige discussie.

I would like to express my gratitude to the colleagues of the Yield Group. The lunch mates for sharing their considerable and trivial knowledge, their humor and every-day life. The lab mates for keeping up the good atmosphere with appropriate music and ‘art’ work, and the party and drinking mates that made many get-togethers worthwhile to remember, with or without the need for beer cards. Also many thanks to the yielders that contributed scientific insights, practical assistance and help with correcting the thesis. A special thank you goes to Nathalie, whose dedication is admirable, and who provided guidance whenever needed, therby setting an excellent example in scientific research and people management. I would also like to thank my desk mate Megan for being a wonderful crazy friend during our years as PhD students.

Furthermore I would like to thank the chair and the members of the examination committee, for their investments and contributions which stimulated me and led to the improvement of this thesis. I’m also very gratefull for the many scientific collaborations that were indispensable for this work.

Kirsten, Elisabeth en Stephen wil ik graag uitdrukkelijk bedanken voor de goeie babbels, soms ook wel de roddels, en de bangelijke feestjes die een noodzaak waren om het doctoraat de doorstaan. Ook Jelle, Steven, Laurens, Toon, Lieven, Marnik, Wilson en alle andere collega's die steeds van de partij waren op feestjes of tijd maakten voor een praatje in de gang verdienen een dikke dankjewel. Mijn dank gaat tevens uit naar de collega's die onvermoeibaar zorgen voor logistieke steun, IT-gerelateerde hulp, de administratie en hulp bij het publiceren.

Ik ben zeer dankbaar voor de hechte vriendschap van Annemie en Philip en het begrip dat de andere vrienden opbrachten omdat ze me de laatste tijd veel hebben moeten missen. Ook zonder de onvoorwaardelijke steun van mijn familie was me dit nooit gelukt. Een grote Dank U gaat uit naar mijn ouders voor de kansen en goede zorgen die ze me gegeven hebben en naar mijn allerliefste zus die ik voor geen geld van de wereld wil missen en alvast wil bedanken voor de ongetwijfeld fantastische organisatie van het feestje. Tot slot wil ik met heel mijn hart Björn bedanken voor zijn onbeschrijfelijke steun, geduld en hulp ondanks zijn drukke agenda en mijn vervelende kantjes. Dat wij dit samen waarmaken doet me sterk geloven in een toekomst vol liefde. Omdat mijn woorden naar mijn mening tekort schieten, wil ik mijn dankbaarheid uitdrukken met een tekst die hopelijk hetzelfde warme gevoel teweeg brengt als bij mij, toen ik hem voor de eerste maal las tijdens het schrijven van deze doctoraatsthesis.

"Indien ik je dragen kon
over de diepe grachten
van je gesukkel en je angsten heen,
dan droeg ik je,
uren en dagen lang.

Indien ik de woorden kende
om antwoord te geven op je duizend vragen
over leven, over jezelf
over liefhebben en gelukkig worden,
dan praatte ik met je,
uren en dagen lang.

Indien ik vrede in je hart kon planten
door geduldig te wachten en te hopen
tot het zaad van vrede in je openbrak,
dan wachtte ik,
uren en dagen lang.

Indien ik genezen kon wat omgaat in je hart
aan onmacht, ontevredenheid
en onverwerkt verdriet,
dan bleef ik naast je staan,
uren en dagen lang.

Maar ik ben niet groter, niet sterken dan jij,
en ik weet niet alles en ik kan niet zoveel,
ik ben maar een vriend op je weg
al uren en dagen lang.

En ik kan alleen maar hopen dat je dit weet :
je hoeft nooit alleen te vechten of te huilen
als je een vriend hebt
voor uren en dagen lang."

- *Marcel Weemaes* -

Veel liefs,
Liesbeth

Curriculum Vitae

Liesbeth Vercruyssen

Contact information

Personal:

Nieuwewandeling 16
9000 Gent
Belgium
Tel: +32 473 33 60 69

Professional:

VIB - Plant Systems Biology
Technologiepark 927
9052 Zwijnaarde
Belgium
Tel: + 32 9 331 39 54
Email: licru@psb.vib-ugent.be

Research:

- Phd research: Integration of cytokinin responses and chromatin remodeling to regulate transcription during Arabidopsis shoot development (Promoter: Prof. Dr. Dirk Inzé)
- Master's research: Analysis of ethylene signalization and biosynthesis genes during nodulation of the tropical legume plant *Sesbania rostrata* (Promoter: Prof. Dr. Marcelle Holsters; Co-promoter: Prof. Dr. Sofie Goormachtig)

Education:

2007-present	Phd in Biotechnology Ghent University/Flanders Institute for biotechnology (VIB) Department of Plant Systems Biology Technologiepark 927, 9052 Zwijnaarde, Belgium
2004-2006	Master in Biotechnology Ghent University Faculty of science Magna cum Laude
2002- 2004	Bachelor in Biology Ghent University Faculty of science Cum Laude

Publications:

L. Vercruyssen, N. Gonzalez, T. Werner, T. Schmülling, and D. Inzé (2011) Combining enhanced root and shoot growth reveals crosstalk between pathways that control plant organ size in Arabidopsis. Plant Physiol. 155: 1339-1352.

Posters & Presentations:

Presentation: L. Vercruyssen, A. Verkest, M. Dubois, N. Gonzalez, L. De Milde, G. De Jaeger and D. Inzé. Chromatin remodeling SWI/SNF complexes: central players in transcriptional control at the switch of developmental programs. Flanders Institute for biotechnology (VIB) Seminar, April, 2012, Blankenberge, Belgium.

Poster: L. Vercruyssen, N. Gonzalez, T. Werner, T. Schmülling, and D. Inzé. Combining enhanced root and shoot growth reveals crosstalk between pathways that control plant organ size in Arabidopsis. Annual Plant Biology Meeting of the American Society of Plant Biologist (ASPB), August, 2011, Minneapolis, Minnesota, USA.

Poster: L. Vercruyssen, N. Gonzalez, T. Werner, T. Schmülling, and D. Inzé. Combining enhanced root and shoot growth reveals crosstalk between pathways that control plant organ size in Arabidopsis. XVII Congress of the Federation of European Societies of Plant Biology (FESPB), July, 2010, Valencia, Spain.

Presentation: L. Vercruyssen, N. Gonzalez, T. Werner, T. Schmülling, and D. Inzé. How can we learn more about plant growth? Boosting the yield of Arabidopsis. Inter-University Attraction Poles (IUAP) meeting, January, 2010, Liège, Belgium.

Presentation: L. Vercruyssen, N. Gonzalez, T. Werner, T. Schmülling, G.T.S. Beemster, and D. Inzé. How can we learn more about plant growth? Boosting the yield of Arabidopsis. Inter-University Attraction Poles (IUAP) meeting, January, 2009, Antwerp, Belgium.

Teaching Experience:

2009-2011	Practical courses Plant Physiology (Bachelor Biology) Supervision of master students
2007-2008	Practical courses Molecular Genetics (Bachelor Biology) Supervision of master students

Professional Memberships:

- European Plant Science Organisation (EPSO)